AD_____

Award Number: DAMD17-02-1-0177

TITLE: Non-Immunogenic Structurally and Biologically Intact Tissue Matrix Grafts for the Immediate Repair of Ballistic-Induced Vascular and Nerve Tissue Injury in Combat

PRINCIPAL INVESTIGATOR: Nathanial Bachrach, Ph.D.

CONTRACTING ORGANIZATION: Lifecell Corporation
Branchburg, New Jersey 08876

REPORT DATE: December 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 2004	Annual (15 Nov 2003 - 14 Nov 2004)

4. TITLE AND SUBTITLE
Non-Immunogenic Structurally and Biologically Intact Tissue Matrix Grafts for the Immediate Repair of Ballistic-Induced Vascular and Nerve Tissue Injury in Combat

5. FUNDING NUMBERS
DAMD17-02-1-0177

6. AUTHOR(S)
Nathanial Bachrach, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Lifecell Corporation
Branchburg, New Jersey 08876

E-Mail: nbachrach@lifecell.com

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
No abstract provided.

14. SUBJECT TERMS
Vascular graft, freeze-dried, umbilical, biological activity, repopulation, patency, arterio-venous shunt, nerve graft

15. NUMBER OF PAGES
74

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....5

Key Research Accomplishments.....12

Reportable Outcomes.....12

Conclusions.....13

References.....14

Appendices.....15

I. Introduction:

In the first two years of this work the production of decellularized and freeze-dried vascular grafts from human umbilical vessels was accomplished while substantially maintaining tissue microstructure biological activity and biomechanical properties. It was anticipated that umbilical vein and artery grafts implanted clinically would demonstrate patency as well as significant repopulation and remodeling by the surrounding host tissue. Such a graft would be a desirable alternative to current interventions for hemodialysis access; occluding non-remodeling synthetic PTFE grafts and inconsistent native fistulas, below-the-knee vessel replacement; amputation, and coronary artery bypass grafting.

The present year focused on several key areas.

- Evaluation of the patency and biological response to the graft in a primate model.
- Development of a shipper for effective low temperature shipment of the frozen umbilical vein graft.
- Evaluation of alternative preservation methods to allow for non-frozen storage.
- Application of gamma irradiation to the tissue to provide some level of microbial reduction. As adverse events have occurred clinically for human tissue products this is a new and essential design aspect for aseptically processed tissues. This included an evaluation of the impact the treatment has on the tissue's biomechanical integrity and biological activities.

The results of this research are summarized below under the appropriate objective heading. Detailed reports are provided as appendices.

II. **I.Objective 1:**
Optimize procurement and processing protocols for umbilical vessels to obtain acellular grafts that retain matrix integrity and biological activity.

This objective was completed last year.

III. Objective 2:
Establish the biomechanical characteristics of these unique, biologically intact umbilical vessel grafts in failure mode analysis.

The evaluation of the frozen version of the graft was completed and reported on in the 2003 Annual Report. Essential design modifications identified in that report included, bioburden reduction through a cold gamma irradiation, development of a non-damaging freeze-drying protocol and an evaluation of alternative preservation methods. These significant design modification activities as well as shipper design for frozen tissue grafts are presented in the following sections.

Shipper Design for Frozen Graft Configuration

The development engineers at LifeCell Corporation designed and tested a series of prototype shippers that would be capable of supplying the Umbilical Vein Graft in its current frozen configuration to the surgical site. The Initial development of a dry ice shipper had been limited to maintainance of temperature lower than -50°C for 24 hours. However, the performance specifications was <-65°C for a minimum of 72 hours. Continued development was contracted to Mocon (Minneapolis, MN) to expand on the design and provide an insulated shipping container capable or meeting those specifications. Also the shipper was to protect the frozen UVG from damage during transit. The design of the shipper and its preliminary testing verification study reports are on file at LifeCell Corporation. The shipper was then tested by an following ISTA (International Safe Transit Association) procedures for packaging and temperature maintenance at Whitehouse Analytical Laboratories, LLC (Whitehouse, NJ). The report is attached as Appendix A.

Cold-Gamma Irradiation of Umbilical Tissue

Before and/or After Processing

A study was conducted to evaluate the potential to increase the safety profile of the human umbilical vein graft (UVG) through gamma irradiation (Appendix B). Since gamma irradiation is known to affect the structural and biological properties of tissues a cold gamma scheme was developed to try and avoid some of the damaging effects of the radiation. An initial study was designed to determine the effect of different γ-irradiation schemes on the following: processing (i.e. difficulty of dissection, pliability, etc), X-101 penetration, histology, electron microscopy, suture retention, circumferential tensile compliance, and test to failure.

Seven umbilical veins were each divided into the following conditions:
W: Control, no γ-irradiation
X: pre-dose of 12 kGy γ-irradiation
Y: final dose of 12 kGy γ-irradiation
Z: pre-dose of 12 kGy γ-irradiation + final dose of 12 kGy γ-irradiation
Samples were packaged for γ-irradiation as per the protocol and stored at approximately -80°C until the time of testing. The in vitro characterization data from both the mechanical testing (Table 1) and the histological analysis (data not shown here) show that no significant differences between any of the treatments.

Table 1: Mechanical Testing Data (n=5; avg ± std dev)

Treatment	Suture Retention Strength (N)	Compliance (N/%strain)[1]	Break (N)
W: Control, no γ-irradiation	4.39 ± 1.91	6.08 ± 1.58	18.95 ± 7.49
X: pre-dose of 12 kGy γ-irradiation	4.28 ± 0.80	9.21 ± 5.08	15.83 ± 5.66
Y: final dose of 12 kGy γ-irradiation	4.95 ± 3.06	7.56 ± 3.48	16.66 ± 6.76
Z: pre-dose of 12 kGy γ-irradiation + final dose of 12 kGy γ-irradiation	4.25 ± 0.90	8.94 ± 8.99	16.05 ± 4.07

Reduced rehydration was observed in the post-dose (Y) and double-dosed (Z) conditions (Table 2). This appears to verify a visual observation that the Wharton's Jelly appeared shrunken and collapsed.

Table 2: Rehydration Data

Treatments	Packaging method before rehydration analysis	Hydration after γ treatment (g water per g dry tissue) *	Hydration after full rehydration (g water per g dry tissue)
W	Tyvek/Foil bag	15.6 ± 1.9	25.0 ± 1. 9
X	Tyvek/Foil bag	12.6 ± 0.9	16.7 ± 1.2
Y	Tyvek/Cassette	19.0 ± 1.6	16.7 ± 0.7
Z	Tyvek/Cassette	13.3 ± 0.3	14.5 ± 1.1

* Data represent the mean ± standard deviation of four measurements (two samples from each of two lots)

This study provided some clues about the effect of γ-irradiation on the umbilical vein graft. However, it is difficult to judge which treatment scheme can be considered optimal. Also, as the level of damage to the tissue seemed negligible a follow-up study in which higher γ-irradiation dosages are utilized was pursued.

Dosing Evaluation

In vitro

A method for maintaining cold (-80°C) temperatures during γ-irradiation was shown to provide a degree of protection of the tissue from damage. A study was undertaken to determine the dose response of a cryoprotected and frozen graft to γ-irradiation at different dosages (0 – control, 12, 18, and 24 kGy). It has also been found that the damaging effects of gamma irradiation can some times be seen in terms of the stability of the tissue with changes occurring over time. Therefore samples were also evaluated for the effect of ambient temperature storage (30 days)

[1] For the compliance test, three samples were tested from each graft. The average of the three measurements for each of the five grafts was then used to generate the data reported in Table 1.

on non-cryoprotected grafts that had been γ-irradiated at 12 kGy. The storage solution can also play a role as a gamma protectant so an additional objective was to determine the equivalence of ambient temperature stored, non-cryoprotected grafts to cryoprotected grafts stored at -80°C (eg storage solution). After irradiation the grafts were evaluated for qualitative changes (color, handling etc), histological changes in the tissue's structure and the mechanical properties of the vessel.

A complete description of the study and the results are presented in Appendix C. The conclusions are summarized here.

Gamma irradiation appeared to have the most effect on tissue handling characteristics. Although γ-irradiated and ambient stored cryoprotected grafts became yellower and darker in color after 30 days, the color change did not appear to have an effect on the mechanical testing results. Histological analysis showed that storage at -80°C in PBS for both γ-irradiated and non-irradiated samples produced more damage to the collagen fibers and created holes in the Wharton's Jelly. The γ-irradiation dosage does not affect the mechanical properties of cryoprotected grafts at time 0.
The use of PBS (ambient storage) did not produce significant changes in mechanical properties compared to cryoprotected grafts at time 0. Ambient storage conditions for cryoprotected grafts did not appear to affect the mechanical properties. Both storage time and □-irradiation at 12 kGy, affected the mechanical properties of non-cryoprotected samples – ie □-irradiation reduced compliance by 55%, storage time reduced load on break by 24%, and there was an interaction in which suture strength dropped by 66% with storage time for non-irradiated samples.

In vivo

After promising results with cold gamma demonstrated that there is minimal damage to the tissue in the in vitro evaluations of the tissue a study was undertaken to evaluate the biological response to these tissues. A nude rat implant model was used to assess the biocompatibility of human umbilical vein grafts (UVG) processed under different conditions. Each rat received four subcutaneous implants: a positive control (X-101, frozen UVG), a negative control (ePTFE), and two of six test conditions. The six test conditions were frozen X-101 grafts irradiated at either 12, 18, or 24 kGy post-processing; frozen X-101 irradiated both pre- and post-processing for a cumulative 24 kGys dosage; ambient PBS stored grafts irradiated post-processing at 12 kGys; and non-irradiated freeze-dried grafts (Table 3). Each test condition was implanted in five rats.

Table 3: Test Articles Implanted

Test Article ID and Lot #	Storage Temperature (°C)	Storage Solution, Condition	Gamma Irradiation	
			Pre- or Post-Processing	Dosage (kGy)
A, 13881 (positive control)	-90° to -65°	X-101, frozen	N/A	0
B, AFB03OGL (negative control)	Ambient	N/A	N/A	N/A
C, 13434	-90° to -65°	X-101, frozen	Post	12
D, 13442	-90° to -65°	X-101, frozen	Post	18
E, 13434	-90° to -65°	X-101, frozen	Post	24
F, 13947A	-90° to -65°	X-101, frozen	Pre + post	12 + 12 = 24
G, 13881	Ambient	PBS	Post	12
H, 13125	1° to 10°	X-101, freeze-dried	N/A	N/A

All rats were euthanized after 21 days' implantation. The explants were each photographed, fixed, and stained for histophathological analysis. The overall finding was that there was a similar response to the test articles compared to the positive control as measured by encapsulation, fibroblast infiltration, neovascularization, and mononuclear cell infiltrates. A quantitative ranking of the conditions yielded relative trends. Compared to the positive control, the single and cumulative dose of 24 kGy conditions were slightly more biocompatible by this scoring methodology while the freeze-dried, PBS, and 18 kGy conditions were less biocompatible. The full study report is on file at LifeCell Corporation (LCP2004-04-03).
This result probably reflects the loss of tissue stability with gamma irradiation that allows faster cell infiltration. This raises the possibility that the tissue that is irradiated beyond 18kGy is damage in a way that could be detrimental in the sense that accelerated remodeling can be catastrophic. A functional tissue substitute, like the UVG, that is expected to regenerate and remodel into host tissue is required to maintain its functional mechanical properties during that transition.

Solution Preservation

Development of Preservation Methods

The preservation of umbilical vein grafts is currently conducted with X-101 that allows freezing the grafts to -80°C for storage. In addition to the -80°C storage, considerations are being made toward using a solution capable of preservation at either room temperature or under minimally refrigerated condition (e.g. 4°C). This is a critical design modification as the objective of this work is to develop an off the shelf graft more suitable for combat casualty care. The two choices evaluated thus far are the use of Glycerol (Gly) and Ethylene Glycol (EG) to substitute water in the tissue matrix. The first step in evaluating these components was to develop a process through which systematic dehydration could occur without damaging the tissue matrix and upon washing in saline, complete rehydration could follow. A number of experiments were performed to develop a process for each of the solutions. The data on the ultimately suggested processes are presented in Appendix D.

For both glycerol and ethylene glycol, each equilibration step can be standardized to a processing time of 1 hour ± 15 minutes. The glycerolization process requires a series of 4 increasing concentrations of 40%, 55%, 70%, and finally 85% while ethylene glycol only requires 2 concentrations of 50% and 90%.
After stagnate rehydration of 40 minutes in 10ml saline/cm on the bench top, samples contained ~18% v/v residual glycerol or ~24% v/v ethylene glycol residual. Finally, the amounts of residual moisture in the samples were 12.2% w/w for glycerol and 10.3% w/w for ethylene glycol.

These studies show that glycerol and ethylene glycol are both capable of water replacement for preservation of Umbilical Vein Grafts. These results led to studies to evaluate the effect that these solutions have on the tissue matrix before introduction into the umbilical vein graft manufacturing process. This information would also provide guidance on which solution would be preferable.

Comparison of Preservation Methods In Vitro

An evaluation was performed to compare the two new preservation methods to the current method of frozen preservation in a cryoprotectant. Three cords were dissected then divided in 3 equal pieces of ~13-15 cm each and processed separately until the preservation step as per standard operating procedures (SOP). Each piece was subjected to one of the following three treatments.

- X-101 treatment followed SOP with one 4 hour ± 10 minute incubation (~4 hour) followed by a second 4 to 24 hour incubation (~19 hours)
- Glycerol treatment consisted of a series of 4 incubations with increasing concentrations of 40%, 55%, 70%, and 85% glycerol v/v with saline at 10ml/cm for 1 hour ± 15 minutes each under constant agitation of 85rpm.
- Ethylene Glycol treatment consisted of a series of 2 incubations with increasing concentrations of 50% and 90% ethylene glycol v/v with saline at 10ml/cm for 1 hour ± 15 minutes each under constant agitation of 85rmp.

Visual observations and measurable characteristics demonstrated that the different treatments do seem to have varying effects on the tissue; however, tissue to tissue variability was also observed. Even though the three treatments leave the tissue with a slightly different appearance, upon re-hydration they all look the same.

Histologically, X-101, glycerol and ethylene glycol had similar evaluations for holes, collagen damage, and collagen separation / orientation for all three sample lots and treatments appeared similar. Also for all sample lots and treatments the basement membrane and internal elastic lamina was present. Lastly no MHC I or II was detected on any of the slides. The only mechanical difference detected between the different treatments was the reduced compliance (N/%) in the Glycerol preserved compared with the X-101 frozen tissue.

Based on the comparison criteria of histology, circumferential, suture retention and burst strength testing the data show that the different processing methods are comparable. The full study report is provided as Appendix E.

Comparison of Preservation Methods In Vivo

The objective of the study is to assess the biocompatibility of tissues preserved in glycerol or ethylene glycol relative to the current cryopreservation method. In addition both 4 degree and 37 degree storage of the samples was tested (Appendix F). The higher temperature storage would provide an early evaluation of an accelerated storage response. Biocompatibility was determined as well integrated tissue that is populated by non-inflammatory host cells and supported by neovascularization of the tissue following implant. Based on this criteria, the test articles and control samples were ranked from most biocompatible (a score of 1) to least biocompatible (a score of 4) by the pathologist. The various test articles are shown in the table below.

Sample	Tissue Type	Storage Conditions
A	Positive control article	-65° to - 90°
B	Negative control article	ambient
C	Test Article – glycerol	4°C ± 1°C
D	Test Article – glycerol	37°C ± 1°C
E	Test Article – ethylene glycol	4°C ± 1°C
F	Test Article – ethylene glycol	37°C ± 1°C

The data showed that the morphology of the host tissue response was similar for the tissue stored in Glycerol and Ethylene Glycol compared to the cryopreserved tisse. The vein grafts were infiltrated by fibroblasts and new blood vessels indicating a favorable biocompatible response and the potential for host cell regeneration and homeostasis of the tissue. The degree of response varied as is to be expected in this type of qualitative evaluation. An attempt was made to assign biocompatibility scores to the samples and then compare in a semi-quantitative analysis. These evaluations were made by the pathologist and are presented along with a study report in Appendix F. The intensities of alterations varied somewhat between each test article as well as between some test articles and the positive control umbilical vein grafts. Test articles stored at 4° C gave similar response to the positive control, while test articles stored at 37° C had less biocompatibility compared to the positive control with a slight increase in the fibroblast infiltration and neovascularization. It is concluded from this study that the acute biocompatibility of the UVG stored in glycerol or ethylene glycol is acceptable. More studies are necessary to fully evaluate the stability of the tissue over time at different temperatures.

Stability evaluation

A glycerol preservation shelf life study was initiated to evaluate the characteristics listed above with an additional test of load at break (N) after multi-puncturing over time. Samples were tested at ~0, 1, and 3 months and were stored at either 4°C or 20°C. The results indicate that histologically over 3 months the samples nay be seeing a change with increased collagen damage orientation and separation. Histological evaluation is a highly variable and the histological methods have not been validated for working with tissue that may contain residual levels of glycerol so this data is not yet conclusive. Mechanically, on the other hand, the data showed tensile strength (load at break and compliance), multi-puncture and the burst strength remained constant over time. The full study report is provided in Appendix G.

IV. Objective 3:

Demonstrate that umbilical vessel grafts transplanted, as carotid interposition implants, in an animal model system maintain patency in the absence of dilatation, aneurysm formation or neointimal hyperplasia.

The 2003 report described the failure to provide appropriate carotid grafts from the umbilical arteries. No modifications in methods of procurement or dissection were successful in providing more robust tissue grafts that warranted re-evaluation in vivo.

V. Objective 4:

Demonstrate in an animal model that these umbilical vessel matrix grafts maintain patency in clinically relevant application simulating long length with low flow dynamics.

The 2002 annual report described the severe inflammatory response that was elicited by the umbilical vein graft when implanted in a porcine model for approximately two weeks. In the 2003 report the data was provided to establish that the reaction seen in that study was a cross-species immune response. While this kind of response to the tissue is a strong indication that the matrix components are well preserved as per the objective of the project, this leaves a lack of compelling data regarding the clinical utility of the graft. A primate model was developed to try

and avoid cross-species response to the grafts and provide longer term data on the repopulation of the umbilical vein graft and its potential to remain patent in vivo.

Initial Primate Study

Human Umbilical Vein Grafts were implanted in large male vervets (one per animal) as an AV shunt from the Iliac/femoral artery to the femoral vein. Needle access was planned as a possible endpoint to demonstrate the potential for a chronic multi-stick model. Grafts were implanted for 2 and 6 weeks, three for each time point, with 3 additional grafts included with an unplanned duration. Synthetic control grafts were also implanted (one graft for each time point). Serum was obtained prior to and during the study to evaluate the immune response of the animal to the graft. The study demonstrated that the umbilical vein graft (UVG) can be implanted as an arteriovenous shunt from the iliac artery to the femoral vein in this primate and remain patent for as long as six weeks. The data showed that there is a humoral immune response to the graft in this model. However, despite this response, no mural degeneration or aneurysms in the graft were noted within the first six weeks. An initial inflammatory reaction to the graft was shown to resolve over time giving way to a healing response and the initiation of new tissue growth. Taken together these results indicate that although this is a xenogeneic model (Human to Monkey), it may provide a means of obtaining information about the expected function, hemocompatability and healing response of the UVG for as long as six weeks in vivo. This study also demonstrated that the graft can be cannulated in the chosen position and that hemostasis after cannulation of the UVG compares favorably to ePTFE. The study report is attached (Appendix H).

Chronic Primate Study

Based on the success of the animal model an additional study was designed to obtain chronic patency data. The initial implant phase of the study was successful. However, there were a series of occlusions noted early in the evaluation of the grafts that indicated that there had been surgical complications. Further evaluation of the implants indicated that more grafts were becoming occluded. Final reporting on the study including pathology from these grafts and the immunologic findings are still in progress.

VI. Objective 5:
Optimize procurement and processing protocols for nerve tissue to obtain an acellular graft that retains matrix integrity and biological activity.

No further progress has been made this past year toward Objective 5.

VII. Objective 6:
Demonstrate repopulation of these unique, biologically intact nerve matrix grafts and restoration of neural function in a clinically relevant nerve defect model system.

No further progress has been made this past year toward Objective 6.

VIII. Key Research Accomplishments:

- Developed dry ice shipper capable of providing the human umbilical vein graft in its frozen state without damage for immediate use or on site storage.
- Determined a method for gamma irradiating the umbilical vein for an increased safety profile without causing significant acute damage to the tissue as evidenced by in vitro and acute subdermal implant evaluations.
- Developed two alternate preservation methods to allow higher temperature storage and shipping for improved functionality.
- Established the biomechanical profile for the grafts preserved by different methods and their suitability for clinical use.
- Established the biocompatibility of the vein grafts in acute small animal models.
- Established the stability of the vein graft for as long as 3 months.
- Developed a primate model to evaluate the response biological response to the graft under arterio-venous flow conditions for up to 6 weeks.
- Demonstrated in a primate model that the vein graft is capable of being sewn in place and maintaining patency for up to 6 weeks. And that during that time the tissue begins to become remodeled by the animal and is well integrated into the adjacent vessels.

IX. Reportable Outcomes:

N/A

X. Conclusions:

The production of decellularized and freeze-dried vascular grafts from human umbilical vessels was accomplished while substantially maintaining tissue microstructure biological activity and biomechanical properties. The data show that it is possible to preserve this tissue matrix and its bioactivity without compromising its mechanical integrity through the use of water removal methods and molecules like ethylene glycol and glycerol. It is also clear that some level of Bioburden reduction is possible with this non-sterile human derived graft that can provide additional safety to the patient. As adverse events have occurred clinically with human tissue products it is becoming an essential design aspect for aseptically processed tissues to provide some level of microbial reduction. This is an area of intense research and the ability to provide sterile tissue products with intact matrix bioactivity would be an essential aspect to providing these much needed regenerative tissue products to patients with critical unmet needs. The off the shelf preservation methods developed combined with this Bioburden reduction provide a significant improvement to the frozen configuration.

It has been demonstrated now that the umbilical vein grafts implanted for up to six weeks in a non-human primate can remain patent and begins to integrate with the host tissue. Unfortunately, ongoing evaluation in the primate model have shown that the model seems to be limited due to mismatch of vessel size and other technical considerations so that more than six weeks data are not obtainable at this time. Still, this data provides significant basis for the expectation that as a long-term clinical implant the graft could demonstrate patency as well as significant repopulation and remodeling by the surrounding host tissue. Such a graft in the off the shelf configuration described in this report would be a desirable alternative to current interventions for hemodialysis access; occluding non-remodeling synthetic PTFE grafts and inconsistent native fistulas, below-the-knee vessel replacement; amputation, and coronary artery bypass grafting.

The progress made in this effort provide a substantial basis for the advancement of these processed vascular tissue grafts as candidate grafts for vascular combat casualty care.

References:

None

Appendix – A
Dry Ice Shipper Design and Testing

1.0 Introduction/Background

1.1 This report summarizes the testing performed by Whitehouse Analytical Laboratories as part of the qualification of a dry ice shipper for the umbilical vein graft (UVG).

1.2 Initial development of a dry ice shipper had been limited to maintainance of temperature lower than -50°C for 24 hours. However, the performance specifications was <-65°C for a minimum of 72 hours. Continued development was contracted to Mocon (Minneapolis, MN) to expand on the design and provide an insulated shipping container capable or meeting those specifications. Also the shipper was to protect the frozen UVG from damage during transit.

1.3 Testing included following ISTA (International Safe Transit Association) procedures for packaging integrity testing was performed at Whitehouse Analytical Laboratories, LLC (Whitehouse, NJ).

2.0 Objectives

2.1 To evaluate the ability of the Mocon dry ice shipper to 1) protect the frozen UVG from damage and 2) maintain a temperature lower than -65°C for at least 72 hours under simulated transit conditions.

3.0 Materials/Equipment

3.1 10 product dummy packages (10cm glass rods packaged in a Teflon tray in a Tyvek pouch within a foil pouch)

3.2 3 Mocon dry ice shippers, 10434-1, -2, and -3

3.3 dry ice

3.4 additional equipment as described in 3.1 and 3.2

4.0 Methods

4.1 Test units were set up as per Attachment 1 of the protocol (see 3.1) prior to undergoing the tests below.

4.2 Fixed Displacement Vibration Test: Each shipper was subjected to a total of 14,400 vibratory impacts (2 adjacent sides, 48 minutes at 150 cycles/min on each side). Observations were made of the package and product integrity.

4.3 Free Fall Drop Test: Each shipper was dropped from a height of 30" following the sequence described in section 3.1. Observations were made of the package and product integrity.

4.4 Thermal Challenge: Each test unit was subjected to the thermal profile in Table 1 below:

Table 1: Thermal Profile

Temperature (°C)	Cycle Time (hrs)	Cumulative Time (hrs)
22	4	4
35	6	10
30	56	66
35	6	72

At LifeCell's request, the thermal mapping was continued beyond the 72hrs of thermal challenge in order to detect the failure point. Test unit 10434-1 was

maintained at 35°C while test units 10434-2 and -3 were maintained at room temperature.

5.0 Deviations

5.1 At LifeCell's request, the temperature challenge was carried out longer than the protocol specified 72 hours in order to determine the true shelf-life of the shipper.

5.2 The first test unit, container 10434-1, was not subjected to the initial 4 hours room temperature exposure for the 72 hrs thermal challenge. An additional 4 hours was added to the second 35°C cycle, thereby resulting in a more rigorous test of the unit.

6.0 Results and Analysis

6.1 Fixed Displacement Vibration Test: No product damage or content was observed in any of the test units.

6.2 Free Fall Drop Test: Tears were observed in all test units at the corners and edges of the shipper. The product stabilizers broke at the corners for test units 10434-1 and -2. However, there was no product breakage. The product stabilizers breakage can be addressed by either using stronger Styrofoam or using the same material as the foam blocks in future designs.

6.3 Thermal Challenge: All units met and exceeded the design specifications.

6.4 The results of the testing are summarized in Table 2 below:

Table 2: Summary of Test Results

Test	Acceptance Criteria	Test Unit		
		10434-1	10434-2	10434-3
Fixed Displacement Vibration	No excessive product movement	Pass	Pass	Pass
	No (simulated) product breakage	Pass	Pass	Pass
Free Fall Drop	No excessive product movement	Pass	Pass	Pass
	No (simulated) product breakage	Pass	Pass	Pass
Thermal Challenge	Temperature <-65°C for ≥ 72hrs	Exceeded, <-65°C for 111 hrs	Exceeded, <-65°C for 138 hrs	Exceeded, <-65°C for 127 hrs

7.0 Conclusions

7.1 The three test units all met or exceeded the acceptance criteria.

7.2 The Mocon dry ice shipper is suitable for use as a 72hrs shipping container for the frozen UVG and that, if necessary, the shipping time can be extended to at least 111 hours without compromising the product.

Appendix – B
Effect of Gamma Irradiation Before and / or After Processing on the Human Umbilical Vein Graft

1. Objective(s)

1.1. To determine the effect of different γ-irradiation schemes on the following: processing (ie difficulty of dissection, pliability, etc), X-101 penetration, histology, electron microscopy, suture retention, circumferential tensile compliance, and test to failure.

2. Protocol

2.1. Seven umbilical veins were each divided into the following conditions:

W: Control, no γ-irradiation
X: pre-dose of 12 kGy γ-irradiation
Y: final dose of 12 kGy γ-irradiation
Z: pre-dose of 12 kGy γ-irradiation + final dose of 12 kGy γ-irradiation

2.2. Samples were packaged for γ-irradiation as per the protocol and stored at approximately -80°C until the time of testing.

2.3. Five out of the seven sets of grafts were processed for testing as described in section 4.5 below. The remaining two were retained

2.4. Prior to testing, samples were removed from storage, the grafts were removed from the packaging and placed into 0.9% NaCl (10ml/cm of graft) After 30 minutes' agitation on a shaking platform (ambient temperature), the saline was drained and replaced, and the grafts placed back on the shaking platform for an additional 30 minutes.

2.5. After the second saline wash, sections of grafts were submitted for testing as per the protocol.

2.6. Due to insufficient sample length for graft 12909, only one set of duplicate measurements could be performed for the X-101 infiltration test, rather than the protocol-specified two measurements on two subsample pieces.

3. Results

3.1. Processing Evaluation

3.1.1. Pre-dose γ–irradiation (treatment X) samples took an additional 30 minutes to thaw in the water bath. The arteries were easier to dissect and did not tear or shred.

3.1.2. Pre-dose γ–irradiation (treatments X and Z) resulted in darker colored grafts compared to no irradiation (W) and post-dose irradiation (Y).

3.1.3. Post-dose γ–irradiation (treatments Y and Z) resulted in collapsed and flattened grafts in which the Whartons Jelly (WJ) had shrunk and was indistinguishable from vein tissue. Although the WJ rehydrated in the usual way, the lumen collapsed during the subsequent handling.

3.1.4. Treatment W samples were found to be slimier than usual and had a foul odor upon rehydration.

3.2. Mechanical Testing

3.2.1. The data from the mechanical tests are summarized in Table 1 below.

3.2.2. The data all fall within the same range regardless of γ-irradiation treatment.

Appendix – B
Effect of Gamma Irradiation Before and / or After Processing on the Human Umbilical Vein Graft

Table 1: Mechanical Testing Data (n=5; avg ± std dev)

Treatment	Suture Retention Strength (N)	Compliance (N/%strain)[1]	Break (N)
W: Control, no γ-irradiation	4.39 ± 1.91	6.08 ± 1.58	18.95 ± 7.49
X: pre-dose of 12 kGy γ-irradiation	4.28 ± 0.80	9.21 ± 5.08	15.83 ± 5.66
Y: final dose of 12 kGy γ-irradiation	4.95 ± 3.06	7.56 ± 3.48	16.66 ± 6.76
Z: pre-dose of 12 kGy γ-irradiation + final dose of 12 kGy γ-irradiation	4.25 ± 0.90	8.94 ± 8.99	16.05 ± 4.07

3.3. X-101 Infiltration

3.3.1. Two random samples from each of the four treatment groups were tested for X-101 infiltration using a thermogravimetric measurement.

3.3.2. No statistical comparisons were made due to the low sample number (n=2) for each treatment. The data, summarized in Table 2 below, show no differences between the four groups.

Table 2: X-101 Infiltration Data

Treatment	Graft ID	%X-101 *
W	12575A	31
	12909A	32
X	12575B	29
	12909B	31
Y	12575A	32
	12909A	32
Z	12575B	30
	12909B	31

* The data represent an average of two measurements for each graft.

3.4. Histology (see section 9.1)

3.4.1. Gamma irradiation did not affect either the internal elastic lamina or basement membrane.

3.4.2. Condensed collagen in the matrix was present in all samples that had been γ-irradiated. However, it was more prevalent in the pre-dissection samples (treatment X).

[1] For the compliance test, three samples were tested from each graft. The average of the three measurements for each of the five grafts was then used to generate the data reported in Table 1.

3.4.3. Post-dissection irradiation (treatment Y) appears to produce more holes in the Wharton's Jelly compared to control (W). However, holes were not observed in those that were double-dosed (Z).

3.4.4. The single-dosed γ-irradiated samples (treatments X and Y) showed more separation compared to the control (W). However, the double-dosed samples (Z) did not appear worse than either X or Y.

3.5. Electron Microscopy (ref. 3.6. above)

3.5.1. The electron microscopy analysis revealed "subtle" differences between the four treatment groups. However, no definitive conclusions could be drawn as to which of the γ-irradiation schemes may be deleterious to the tissue.

3.5.2. Samples in treatment W (the control group) showed a higher level of enthothelial cell debris and cell debris in the empty spaces compared to historical samples. The electron microscopist speculated that the decellularization step may not have been as complete as expected.

3.5.3. Treatment in the pre-dissection samples (X) appears to be better compared to either Y or Z based on basement membrane coverage and spacing/separation of tissue.

3.6. Rehydration

3.6.1. Two samples from each of two lots, 12575A and 12909A, were tested.

3.6.2. Table 3 contains some excerpted data from the report on those tests.

Table 3: Rehydration Data

Treatments	Packaging method before rehydration analysis	Hydration after γ treatment (g water per g dry tissue) *	Hydration after full rehydration (g water per g dry tissue)
W	Tyvek/Foil bag	15.6 ± 1.9	25.0 ± 1. 9
X	Tyvek/Foil bag	12.6 ± 0.9	16.7 ± 1.2
Y	Tyvek/Cassette	19.0 ± 1.6	16.7 ± 0.7
Z	Tyvek/Cassette	13.3 ± 0.3	14.5 ± 1.1

* Data represent the mean ± standard deviation of four measurements (two samples from each of two lots)

3.6.3. The data show that both pre- and post-dose γ-irradiation resulted in an approximately 35% decrease in rehydration compared to the control which was not irradiated.

3.6.4. There was an additional 13% decrease in hydration for the double-dosed grafts (Z) compared to the single-dosed grafts (X and Y).

4. Analysis

A 2-way ANOVA test with replication was performed on the mechanical testing data at $p<0.05$. The factors were the pre- and post-dose γ-irradiation. The results are shown in Table

4 for compliance, Table 5 for break, and Table 6 for suture strength. The data show no significant difference among the treatments.

Table 4: 2-Way ANOVA Results for Compliance (N/% strain)

	Repeated Measures Analysis of Variance compliance Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	1263.89	1	1263.89	35.9195	0.00390
Error	140.75	4	35.19		
PRE-DOSE	25.43	1	25.43	1.0676	0.35987
Error	95.26	4	23.82		
POST-DOS	1.82	1	1.82	0.0486	0.83625
Error	149.51	4	37.38		
PRE-DOSE*POST-DOS	3.85	1	3.85	0.1541	0.71464
Error	99.80	4	24.95		

Table 5: 2-Way ANOVA Results for Break (N)

	Repeated Measures Analysis of Variance break (N) Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	5693.63	1	5693.63	65.3947	0.00127
Error	348.26	4	87.07		
PRE-DOSE	17.50	1	17.50	0.6759	0.45717
Error	103.58	4	25.90		
POST-DOS	5.38	1	5.38	0.4876	0.52347
Error	44.11	4	11.03		
PRE-DOSE*POST-DOS	7.88	1	7.88	0.2986	0.61383
Error	105.51	4	26.38		

Appendix – B
Effect of Gamma Irradiation Before and / or After Processing on the Human Umbilical Vein Graft

Table 6: 2-Way ANOVA Results for Suture Strength (N)

Effect	Repeated Measures Analysis of Variance suture strength (N) Sigma-restricted parameterization Effective hypothesis decomposition SS	Degr. of Freedom	MS	F	p
Intercept	399.08	1	399.08	40.13	0.00
Error	39.78	4	9.95		
PRE-DOSE	0.84	1	0.84	0.26	0.63
Error	12.78	4	3.19		
POST-DOS	0.35	1	0.35	0.40	0.56
Error	3.52	4	0.88		
PRE-DOSE*POST-DOS	0.44	1	0.44	1.05	0.36
Error	1.67	4	0.42		

5. Conclusions

5.1. The pre-dose γ-irradiation appeared to affect the ease of arterial dissection. However, it is not known at this time whether the extended thawing time or the darker colored graft will have a significant effect on functional performance.

5.2. The *in vitro* characterization data from both the mechanical testing and the microscopic analysis show that no significant differences between any of the treatments.

5.3. The reduced rehydration observed in the post-dose (Y) and double-dosed (Z) conditions appears to verify the visual observation that the Wharton's Jelly appeared shrunken and collapsed.

5.4. This study has yielded some clues about the effect of γ-irradiation on the umbilical vein graft. However, it is difficult to judge at this time which treatment scheme can be considered optimal. Therefore, follow-up studies in which higher γ-irradiation dosages are utilized are recommended.

Appendix – B
Effect of Gamma Irradiation Before and / or After Processing on the Human Umbilical Vein Graft

6. Appendices

6.1. Histology Data

Table 7: Histological Evaluation Results

		H & E						
Slide #	**Cord #**	**Group**	**Matrix**	**Holes**	**Separation**	**PAS**	**Verhoeffs**	**Comments**
5392-1	12575	W	A	C	A	B	B	
5392-2	12575	X	C	C	C	B	B	
5392-3	12575	Y	D	D	C	B	B	
5392-4	12575	Z	D	A	C	B	B	
5392-5	12909	W	B	A	B	B	B	
5392-6	12909	X	C	A	D	B	A	
5392-7	12909	Y	A	D	C	B	A	
5392-8	12909	Z	D	B	A	B	B	
5392-9	12799	W	D	B	B	B	B	
5392-10	12799	X	C	A	C	C	B	
5392-11	12799	Y	D	A	C	C	B	
5392-12	12799	Z	C	A	B	B	B	
5392-13	12798	W	A	C	C	B	A	Fell between C & D separation
5392-14	12798	X	C	A	C	B	B	Fell between C & D separation
5392-15	12798	Y	C	A	D	A	A	Fell between C & D separation
5392-16	12798	Z	B	A	D	B	B	Fell between C & D separation, "Blown Apart"
5392-17	12820	W	C	A	A	B	B	
5392-18	12820	X	C	A	C	B	B	Fell between C & D separation
5392-19	12820	Y	C	B	D	B	B	Fell between C & D separation, "Blown Apart"
5392-20	12820	Z	D	A	C	B	B	

Group Descriptions	
W	Control
X	12 kGy Pre-Dose
Y	12 kGy Post-Dose
Z	12 kGy Pre and 12kGy Post Dose

Appendix – B
Effect of Gamma Irradiation Before and / or After Processing on the Human Umbilical Vein Graft

Table 8: Histology Rankings Definition

Description of categories		
Matrix	A	Nothing
	B	Damage (Broken or Torn)
	C	Areas of Condensed Matrix
	D	Both Damaged and Condensed areas
Holes	A	Nothing
	B	In media
	C	Very large in WJ
	D	In both Media and WJ
Separation	A	Typical Control Levels
	B	A little worse, more separation
	C	More throughout the media and into WJ
	D	Everywhere
PAS	A	Complete
	B	In-complete (at least one break)
	C	More In-complete (torn even on H&E)
Verhoeffs	A	Complete
	B	In-complete (split or gaps)

Appendix – C
The Effect of Gamma Irradiation Dosage and Storage on Human Umbilical Vein Grafts

1. Objective(s)

1.1. To determine the dose response of a cryoprotected[1] and frozen graft to γ-irradiation (0, 12, 18, and 24 kGy).

1.2. To determine the effect of ambient temperature storage (30 days) on non-cryoprotected grafts that had been γ-irradiated at 12 kGy.

1.3. To determine the equivalence of ambient temperature stored, non-cryoprotected grafts to cryoprotected grafts stored at -80°C (eg storage solution).

1.4. To evaluate the effect of accelerated storage on cryoprotected grafts stored under ambient conditions.

2. Protocol(s)

2.1. Eleven umbilical cords were processed. The grafts used and associated test conditions are summarized in Table 1, Table 2, and Table 3 below.

Table 1: Cryoprotected Grafts Stored 30 Days

			Time 0, -80°C		D30, ambient	
Lot	Graft ID	Storage Sol'n	A	B	C	D
1	12698	X-101	0	12	0	12
2	12709	X-101	0	18	0	18
3	12936	X-101	0	24	0	24

Table 2: Non-Cryoprotected Grafts Stored at Ambient Temperature

			Time 0		D30	
Lot	Graft ID	Storage Sol'n	A	B	C	D
4	12976	PBS	0	12	0	12
5	12937	PBS	0	12	0	12
6	13380	PBS	0	12	0	12

[1] In the context of this study, "cryoprotected" refers to grafts that have been equilibrated with X-101 (also referred to as PD-30) and stored at -80°C.

Appendix – C
The Effect of Gamma Irradiation Dosage and Storage on Human Umbilical Vein Grafts

Table 3: γ-Irradiation Dosages (kGy), -80°C Storage Samples (Time 0)

			-80^{0}C Storage			
Lot	Graft ID	Storage Sol'n	A	B	C	D
7	13057	PBS	0	12	12	12
8	12406	X-101	0	12	18	24
9	12499	X-101	0	12	18	24
10	12500	X-101	0	12	18	24
11	12708	X-101	0	12	18	24

2.2. Prior to testing, samples were removed from storage, the grafts removed from their packaging and placed into 10ml saline/cm graft. After 30 minutes' agitation on a shaking platform (ambient temperature), the saline was drained and replaced, and the grafts placed back on the shaking platform for an additional 30 minutes.

2.3. After the second saline wash, sections of grafts were submitted for testing as per protocol requirements.

2.4. Rehydration assay: 1 cm samples from the circumferential test were incubated 24 hrs in saline. Each sample was blotted dry and weighed to obtain the initial wet weight. The samples were then vacuum dried at 107°C for 30 hrs, after which the dry tissue weight was obtained. The rehydration was determined as the ratio of g water to g dry weight.

3. Results

3.1. Processing Evaluation (notebook 224-23)

3.1.1. White dots were observed in lots 2, 3, 8, 9, 10, and 11 immediately prior to rehydration and disappeared thereafter. In the cases of lots 8-11, the dots were more prevalent with increasing γ-irradiation dose.

3.1.2. Although no color change was evident immediately after γ-irradiation , after 30 days' storage under ambient conditions, γ-irradiated cryoprotected grafts became darker in color compared to equivalently stored controls. At least for these samples, the darkness of the color change did not appear to be correlated with γ-irradiation dosage.

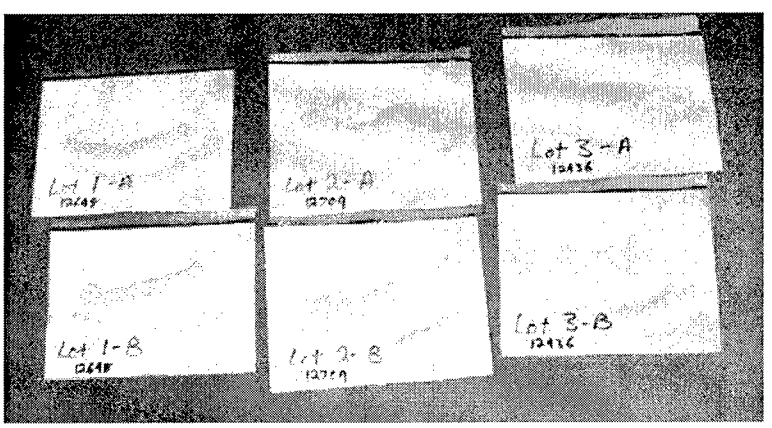


Figure 1a: Time 0; A=control, B=γ-irradiated

Appendix – C
The Effect of Gamma Irradiation Dosage and Storage on Human Umbilical Vein Grafts

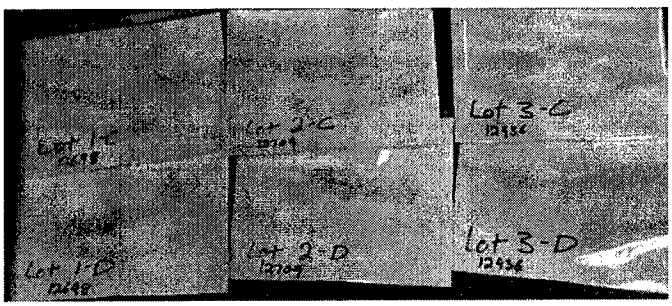


Figure 1b: 30 days; C=control, D=γ-irradiated

Figure 1: Color Change with 30 Days' Storage of Cryprotected Grafts Under Ambient Conditions γ-irradiation dosages: lot 1 = 12 kGy, lot 2 = 18 kGy, and lot 3 = 24 kGy

3.1.3. γ-irradiated grafts were more difficult to cut for histology and electron microscopy sampling.

3.2. Mechanical Testing

The following statistical tests were performed on the mechanical testing data: A 1-way ANOVA was performed to examine the dose response of cryoprotected grafts to γ-irradiation. A 2-way ANOVA was performed to evaluate the effect of ambient storage on non-cryoprotected grafts. An unpaired t-test was used to compare non-cryoprotected (i.e. PBS at ambient conditions) to cryoprotected grafts (-80°C) (e.g. storage solution). A paired t-test was used to evaluate the effect of accelerated storage (ie ambient conditions) on cryoprotected grafts. Statistical significance was set at $p<0.05$ for all analyses.

3.2.1. Suture Strength (N)

3.2.1.1. Suture strength was tested on 2 cm segments from each graft as per RP-0024. The data are summarized in Table 4 for the ambient storage samples and Table 5 for the -80°C samples.

3.2.1.2. Statistical analyses showed no significant differences in suture strength for the following variables: γ-irradiation dosage on cryoprotected grafts, storage solution, and accelerated storage for cryoprotected grafts. There was, however, a significant 2-way interaction between both time and γ-irradiation for the ambient stored non-cryoprotected grafts. That is, the 12 kGy dose did not produce a reduction in suture strength seen with the non-irradiated tissue (see Table 4).

Appendix – C
The Effect of Gamma Irradiation Dosage and Storage on Human Umbilical Vein Grafts

Table 4: Suture Strength (N) (ave ± std dev, (n))
30 Days' Storage Under Ambient Conditions

			γ-Irradiation Dosage (kGy)			
Storage Sol'n	Time Point (days)	Storage Temp (°C)	0	12	18	24
X-101	0	-80	5.34 ± 1.70 (7)	4.00 ± 1.64 (5)	4.25 ± 1.81 (5)	5.03 ± 1.85 (5)
	30	ambient	3.51 ± 1.43 (3)	3.43 (1)	3.50 (1)	5.02 (1)
PBS	0	ambient	5.84 ± 0.83 (3)	3.99 ± 0.99 (3)		
	30	ambient	1.98 ± 1.16 (3)	4.80 ± 1.93 (3)		

Table 5: Suture Strength (N) (ave ± std dev, (n))
-80°C Storage Samples (Time 0)

	γ-Irradiation Dosage (kGy)			
Storage Sol'n	0	12	18	24
PBS	4.31 (1)	3.77 ± 1.24 (3)		
X-101	5.34 ± 1.70 (7)	4.00 ± 1.64 (5)	4.25 ± 1.81 (5)	5.03 ± 1.85 (5)

3.2.2. Compliance (N/%strain)

3.2.2.1. Three 1 cm segments for each graft were subjected to circumferential testing to assess compliance. The data are summarized in Table 6 for the ambient storage samples and in Table 7 for the -80°C samples.

3.2.2.2. Statistical analyses showed no significant differences in compliance for the following variables: γ-irradiation dosage on cryoprotected grafts, storage solution, and accelerated storage for cryoprotected grafts. There was a significant reduction in compliance from γ-irradiation for the ambient stored non-cryoprotected grafts (see Table 6).

Appendix – C
The Effect of Gamma Irradiation Dosage and Storage on Human Umbilical Vein Grafts

Table 6: Compliance Data (N/%strain) (ave ± std dev, (n))
30 Days' Storage Under Ambient Conditions

			γ-Irradiation Dosage (kGy)			
Storage Sol'n	Time Point (days)	Storage Temp (°C)	0	12	18	24
X-101	0	-80	9.87 ± 2.92 (7)	9.19 ± 4.76 (5)	12.25 ± 4.32 (5)	4.80 ± 1.76 (5)
	30	ambient	7.35 ± 4.56 (3)	10.90 (1)	9.28 (1)	4.97 (1)
PBS	0	ambient	9.67 ± 2.14 (3)	4.18 ± 1.25 (3)		
	30	ambient	12.14 ± 3.45 (3)	5.59 ± 1.40 (3)		

Table 7: Compliance Data (N/%strain) (ave ± std dev, (n))
-80°C Storage Samples (Time 0)

	γ-Irradiation Dosage (kGy)			
Storage Sol'n	0	12	18	24
PBS	14.70 (1)	10.28 ± 4.16 (3)		
X-101	9.87 ± 2.92 (7)	9.19 ± 4.76 (5)	12.25 ± 4.32 (5)	4.80 ± 1.76 (5)

3.2.3. Load at Break

3.2.3.1. The load at break data was collected as part of the circumferential test. The data are summarized in Table 8 for the ambient storage samples and in Table 9 for the -80°C samples.

3.2.3.2. Statistical analyses showed no significant differences in load at break for the following variables: γ-irradiation dosage on cryoprotected grafts, storage solution, and accelerated storage for cryoprotected grafts. There was a significant reduction in load at break from storage time for the ambient stored non-cryoprotected grafts (see Table 8).

Appendix – C
The Effect of Gamma Irradiation Dosage and Storage on Human Umbilical Vein Grafts

Table 8: Load at Break Data (N) (ave ± std dev, (n))
30 Days' Storage Under Ambient Conditions

			γ-Irradiation Dosage (kGy)			
Storage Sol'n	Time Point (days)	Storage Temp (°C)	0	12	18	24
X-101	0	-80	22.93 ± 8.92 (7)	16.97 ± 8.54 (5)	18.98 ± 8.36 (5)	16.61 ± 4.87 (5)
	30	ambient	16.16 ± 7.24 (3)	10.90 (1)	19.54 (1)	19.48 (1)
PBS	0	ambient	21.26 ± 1.81 (3)	18.75 ± 1.60 (3)		
	30	ambient	14.84 ± 1.76 (3)	15.62 ± 3.75 (3)		

Table 9: Load at Break Data (N) (ave ± std dev, (n))
-80°C Storage Samples (Time 0)

	γ-Irradiation Dosage (kGy)			
Storage Sol'n	0	12	18	24
PBS	18.57 (1)	14.18 ± 3.05 (3)		
X-101	22.93 ± 8.92 (7)	16.97 ± 8.54 (5)	18.98 ± 8.36 (5)	16.61 ± 4.87 (5)

3.3. Histology (H&E Staining)

3.3.1. With few exceptions, the differences between treatments appear to be subtle. Therefore, no definitive conclusions can be made at this time. The following is a summary of the findings.

3.3.2. Samples, both non-irradiated and γ-irradiated at 12 kGy, that had been frozen in PBS at -80°C exhibited the most damage to the collagen fibers and holes in the Wharton's Jelly.

3.3.3. Thirty days' storage under ambient conditions for both irradiated and non-irradiated samples did not appear to produce additional damage compared to time 0.

3.4. Electron Microscopy

3.4.1. Data were collected for information only and will be reported separately.

3.5. Rehydration (notebook 229:51-54 and 61)

3.5.1. The rehydration data for the 30 days' ambient storage samples are summarized in Table 10 while those for the -80°C time 0 samples are in Table 11.

Appendix – C
The Effect of Gamma Irradiation Dosage and Storage on Human Umbilical Vein Grafts

Table 10: Rehydration Data (ave ± std dev, (n))
30 Days' Storage Under Ambient Conditions

			γ-Irradiation Dosage (kGy)			
Storage Sol'n	Time Point (days)	Storage Temp (°C)	0	12	18	24
X-101	0	-80	29.8 ± 4.2 (7) *	19.0 (1)	14.1 (1)	17.4 (1)
	30	ambient	35.5 ± 2.9 (3)	16.2 (1)	14.4 (1)	15.8 (1)
PBS	0	ambient	25.3 ± 0.9 (3)	13.0 ± 1.8 (3)		
	30	ambient	29.5 ± 2.1 (3)	15.2 ± 1.0 (3)		

* The sample number, n, represents the number of lots tested. Three replicate pieces were tested within each lot.

Table 11: Rehydration Data (ave ± std dev, (n))
-80°C Storage Samples (Time 0)

	γ-Irradiation Dosage (kGy)			
Storage Sol'n	0	12	18	24
PBS	24.2 (1)	12.3 ± 0.6 (3)		
X-101	29.8 ± 4.2 (7)	16.4 ± 3.7 (5)	16.3 ± 3.4 (5)	15.8 ± 2.4 (5)

3.5.2. The combined data in Table 10 and Table 11 show that, for γ-irradiated samples, neither γ-irradiation dosage nor storage time affects rehydration. However, γ-irradiated samples, whether cryoprotected or stored in PBS, do exhibit reduced rehydration compared to non-irradiated samples. Furthermore, the reduced rehydration is similar regardless of the storage solution type.

3.5.3. The data in Table 10 also show an increased hydration for non-irradiated samples that had been stored for 30 days at ambient conditions in either X-101 or PBS. There may be some subtle changes in the matrix structure for the ambient condition stored samples that allowed for the additional hydration.

4. Conclusions

The results of the spectrum study showed the following:

1. Gamma irradiation appeared to have the most effect on tissue handling characteristics, especially for the pre-dose samples. Furthermore, although γ-irradiated and ambient

stored cryoprotected grafts became yellower and darker in color after 30 days, the color change did not appear to have an effect on the mechanical testing results.

2. Histological analysis showed that storage at -80°C in PBS for both γ-irradiated and non-irradiated samples produced more damage to the collagen fibers and created holes in the Wharton's Jelly. It should be noted that there were subtle differences between all samples examined but no conclusive determinations can be drawn at this time.
3. γ-irradiation dosage does not affect the mechanical properties of cryoprotected grafts at time 0.
4. The use of PBS (ambient storage) did not produce significant changes in mechanical properties compared to cryoprotected grafts at time 0.
5. Ambient storage conditions for cryoprotected grafts did not appear to affect the mechanical properties.
6. Both storage time and γ-irradiation (12 kGy) affected the mechanical properties of non-cryoprotected samples – ie γ-irradiation reduced compliance by 55%, storage time reduced load on break by 24%, and a 2-way interaction in which suture strength dropped by 66% with storage time for non-irradiated samples.

Appendix – D
Development of Preservation Methods for Human Umbilical Vein Grafts

1. Objective

To develop dehydration and rehydration procedures for Glycerol and Ethylene Glycol.

2. Results

2.2 Dehydration Process

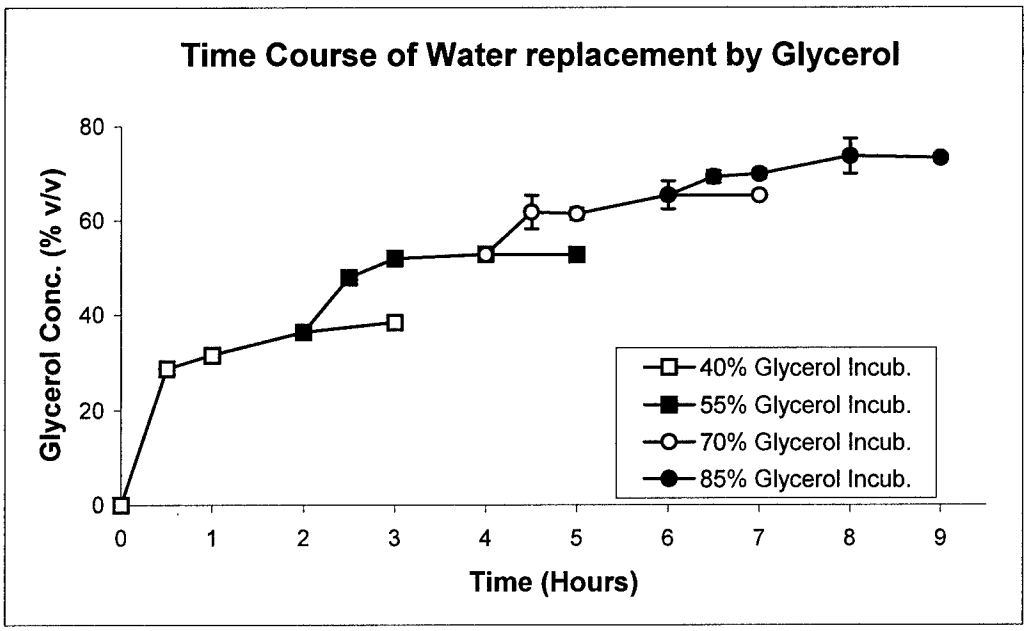
The highest concentration of a solution that would cause the least amount of volumetric change was used to develop the dehydration process. In addition to solution concentrations, the time required for the tissue to reach equilibration at each solution concentration was also determined.

2.2.1 Glycerol (Gly)

Approximately 1 cm pieces of vascular graft were exposed to increasing glycerol (v Gly/v saline) concentrations for two hours at room temperature under constant agitation (85 rpm). The step-wise equilibration was measured using a refractometer (Brix % method).

The equilibration data are shown in Graph 1. The results suggest the following equilibration scheme: 40% v/v Glycerol for 1 hour, 55% for 1 hour, 70% for 1 hour, and 85% for 1 hour. This produces final Glycerol levels of ~73% v/v inside the tissue.

Graph 1) Concentration of Glycerol in tissue matrix during water replacement.

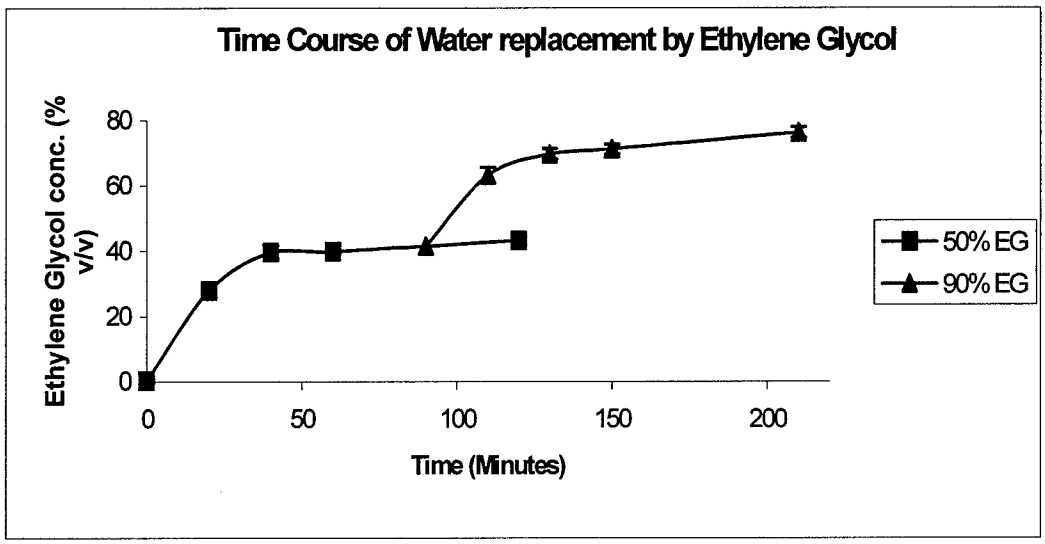


5.2.2 Ethylene Glycol (EG)

Approximately 1 cm pieces of vascular graft were exposed to increasing ethylene glycol (v EG/v saline) concentrations for two hours at room

temperature under constant agitation (85 rpm). The step-wise equilibration was measured using a refractometer (Brix % method).
The equilibration data are shown in Graph 2. The results suggest the following equilibration scheme: 50% v/v EG for 40 minutes and 90% for 40 minutes. This produces final Ethylene Glycol levels of ~71% v/v inside the tissue.

Graph 2) Concentration of Ethylene Glycol in tissue matrix during water replacement.



2.3 Rehydration process

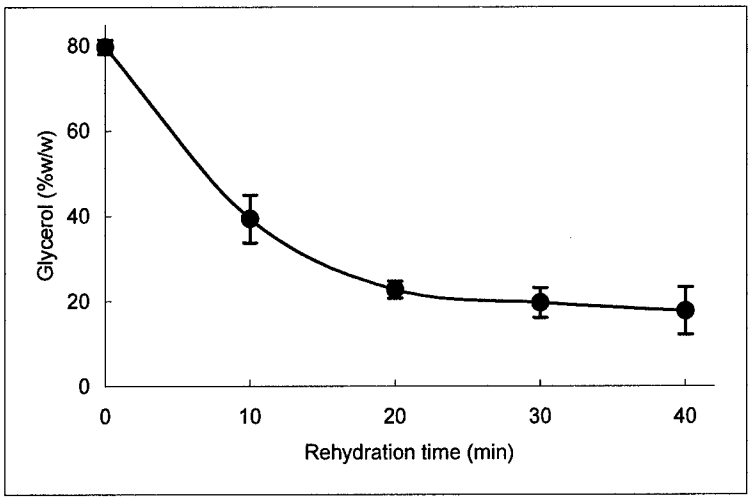
Determined the concentration of Glycerol or Ethylene Glycol remaining in graft after stagnate rehydration in 10 ml saline/cm for different durations.

2.3.1 Glycerol (Gly)

Glycerol concentrations decreased rapidly during the first 20 minutes to ~22% v/v. Then slightly decreased until 40 minutes rehydration where ~18% v/v glycerol remained in the tissue (see graph 3).

Graph 3) Glycerol Concentration in graft during re-hydration

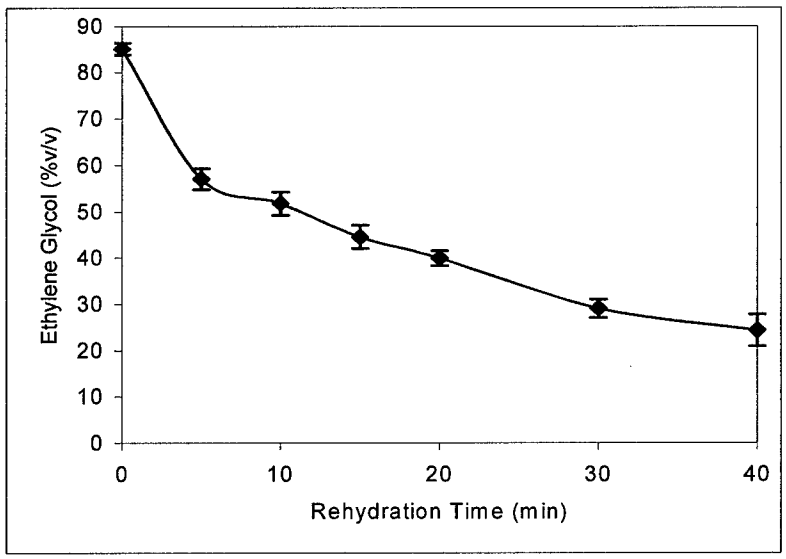
Appendix – D
Development of Preservation Methods for Human Umbilical Vein Grafts



2.3.2 Ethylene Glycol (EG)

Ethylene Glycol concentrations decreased slower than the glycerol treated grafts and after 40 minutes of rehydration, ~24% v/v ethylene glycol remained in the tissue (see graph 4).

Graph 4) Ethylene Glycol Concentration in graft during rehydration



2.4 Residual Moisture in the treated graft

Residual moisture is the water content (% w/w) in the graft after the water replacement treatment.

2.4.1 Glycerol

Residual moisture was determined to be 12.2% w/w.

2.4.2 Ethylene Glycol

Residual moisture was determined to be10.3% w/w.

3. Analysis

For both glycerol and ethylene glycol, each equilibration step can be standardized to a processing time of 1 hour ± 15 minutes. The glycerolization process requires a series of 4 increasing concentrations of 40%, 55%, 70%, and finally 85% while ethylene glycol only requires 2 concentrations of 50% and 90%.
After stagnate rehydration of 40 minutes in 10ml saline/cm on the bench top, samples contained ~18% v/v residual glycerol or ~24% v/v ethylene glycol residual. Finally, the amounts of residual moisture in the samples were 12.2% w/w for glycerol and 10.3% w/w for ethylene glycol.

4. Discussion

Glycerol and ethylene glycol are both capable of water replacement for preservation of Umbilical Vein Grafts. However, more data on the toxicology profiles of these molecules will be required prior to their introduction into the umbilical vein graft manufacturing process.

5. Conclusions

More testing and research are required to determine the in vitro and in vivo effects of storage in these solutions before a determination is made regarding which solution may be better for preservation of Umbilical Vein Grafts.

Appendix – E
In Vitro Evaluation of Different Preservation Methods for the Umbilical Vein Graft

1. Objective(s)

To evaluate and compare the histological and mechanical properties of umbilical vein grafts processed either in Glycerol or Ethylene Glycol to those processed and frozen in X-101.

2. Protocol

Three cords (lots 13755, 13581 and 13754) were dissected then divided in 3 equal pieces of ~13-15 cm each and processed through 3rd PBS after DNase as per standard operating procedures (SOP) separately. Each piece was subjected to one of the three treatments.

The X-101 treatment followed SOP with one 4 hour ± 10 minute incubation (~4 hour) followed by a second 4 to 24 hour incubation (~19 hours) of 35% w/w at 10ml/cm each under constant agitation of 100rpm. After processing, X-101 samples were packaged as per SOP in Tyvek and Foil and frozen to -80°C.

The Glycerol treatment consisted of a series of 4 incubations with increasing concentrations of 40%, 55%, 70%, and 85% glycerol v/v with saline at 10ml/cm for 1 hour ± 15 minutes each under constant agitation of 85rpm. The Ethylene Glycol treatment consisted of a series of 2 incubations with increasing concentrations of 50% and 90% ethylene glycol v/v with saline at 10ml/cm for 1 hour ± 15 minutes each under constant agitation of 85rmp. Glycerol and Ethylene Glycol samples were packaged in 60ml bottles with 10ml/cm from the last incubation solution used (85% for Glycerol and 90% for Ethylene Glycol). Glycerol and ethylene glycol samples remained on the bench top at room temperature until time of testing.

Prior to testing, samples were rinsed as follows:

- X-101 samples received 2 30 minute 100rpm agitation washes with 0.9% sodium chloride at 10mL/cm.
- Both Glycerol and Ethylene Glycol samples received a 40 minute stagnant wash in 0.9% sodium chloride after 5-7 inverts at 10mL/cm.

There was one deviation during the performance of the burst test. A burst pressure for the X-101 condition of lot 13755 could not be determined as the test sample kept detaching from the testing apparatus at the higher flow rate.

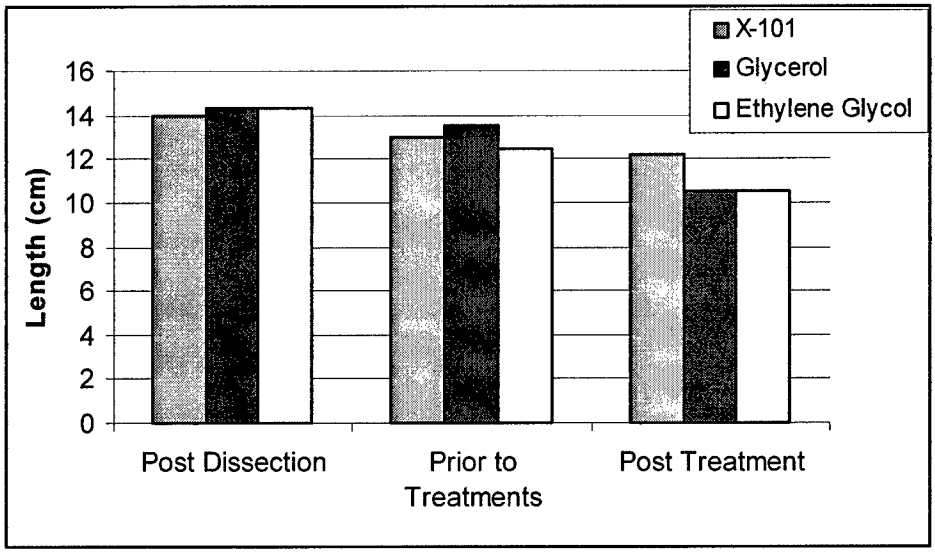
3. Results/Analysis

Visual Observations

Characteristics such as length, number of coils and lumenal diameter were noted post-dissection, and pre- and post-solution treatments. The length of the samples does seem to decrease following dissection with the processing of the tissue and then again with the loading of the preservation solutions (Graph 1).

Graph 1) Changes in length through dissection and treatments

Appendix – E
In Vitro Evaluation of Different Preservation Methods for the Umbilical Vein Graft



After the treatments and prior to packaging samples were again observed for macroscopic differences (see Chart 1).

Chart 1) Visual Appearance after Treatments

Sample ID	Treatment	Appearance	Lumen
13754	X-101	Off white	Closed
	Glycerol	Translucent	Closed
	Ethylene Glycol	Off white	Opened
13581	X-101	Off white	Closed
	Glycerol	Translucent	Closed
	Ethylene Glycol	Off white	Opened
13755	X-101	Off white	Opened
	Glycerol	Only Translucent outside WJ	Opened
	Ethylene Glycol	Off white	Opened

Histology

In general, histological examination revealed X-101, Glycerol and Ethylene glycol samples all contained some amount of nuclear debris. X-101 samples contained the least and it was concentrated close to and in the vein wall, as opposed to the Glycerol and Ethylene Glycol where more debris was evident but scattered throughout the Wharton's Jelly. A matrix evaluation of holes, collagen damage and collagen separation/orientation showed Glycerol and Ethylene Glycol samples are comparable to X-101. The basement membrane and internal elastic lamina were present in all samples and no MHC I or II were detected. (See Chart 2 for details and Figures 1-6 for representative photographs)

Appendix – E
In Vitro Evaluation of Different Preservation Methods for the Umbilical Vein Graft

Chart 2) Histology slide review and scoring

Sample ID	Treatment	Nuclear Debris (Present or Absent)	Holes	Collagen Damage	Collagen Separation/Orientation	Basement Membrane (Present or Absent)	Elastic Lamina (Present or Absent)	MHC I/II (Positive or Negative)
13754	X-101	Present	0	0	0.5	Present	Present	Negative
	Glycerol	Present	1	0	1	Present	Present	Negative
	Ethylene Glycol	Present	1	0.5	0	Present	Present	Negative
13581	X-101	Present	0	0.5	0.5	Present	Present	Negative
	Glycerol	Present	0	1	2	Present	Present	Negative
	Ethylene Glycol	Present	0	0.5	1.5	Present	Present	Negative
13755	X-101	Present	0	0	0	Present	Present	Negative
	Glycerol	Present	1	0	0.5	Present	Present	Negative
	Ethylene Glycol	Present	0.5	0	0.5	Present	Present	Negative

Grading System 0-4		Holes	Collagen Damage	Collagen Separation/ Orientation
	0	None	Intact	No separation
	4	Extreme situation		

The Figures 1-6 below represent sample 13755, focusing on matrix integrity. Note these are representative photographs for all three grafts tested.

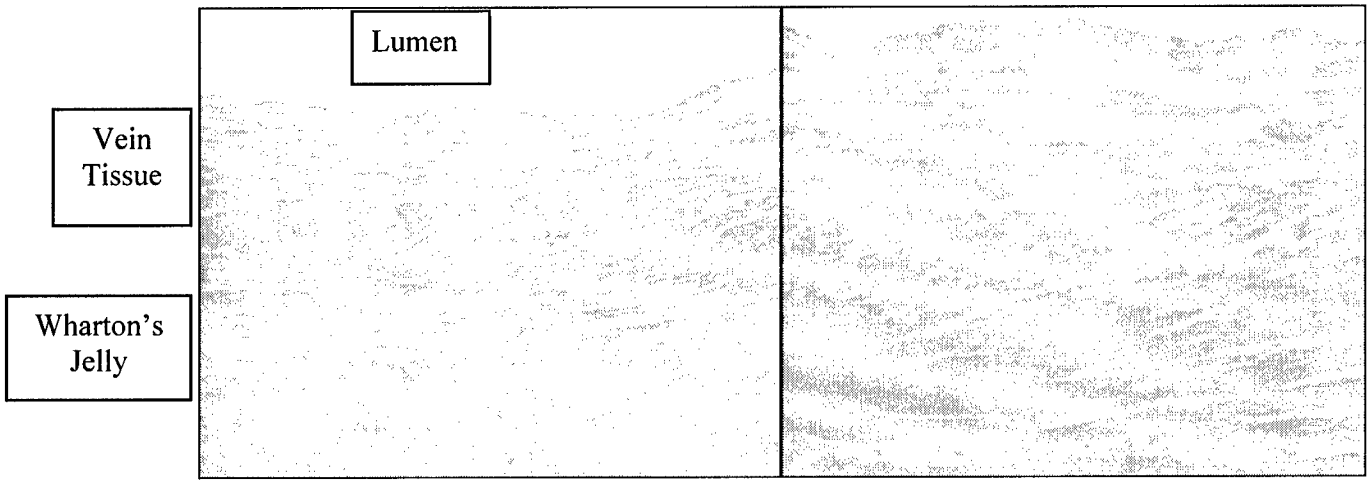


Figure 1) X-101 10X H&E

Figure 2) X-101 20X H&E

Appendix – E
In Vitro Evaluation of Different Preservation Methods for the Umbilical Vein Graft

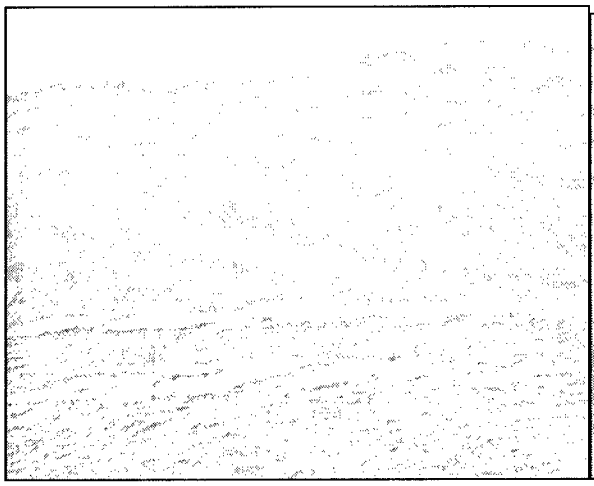


Figure 3) Glycerol 10X H&E

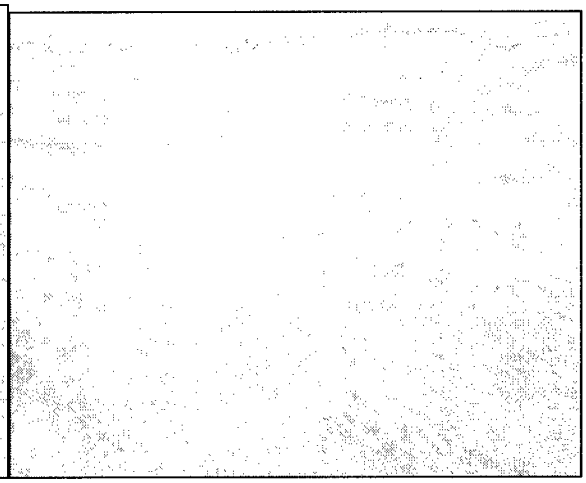


Figure 4) Glycerol 20X H&E

Figure 5) Ethylene Glycol 10X H&E

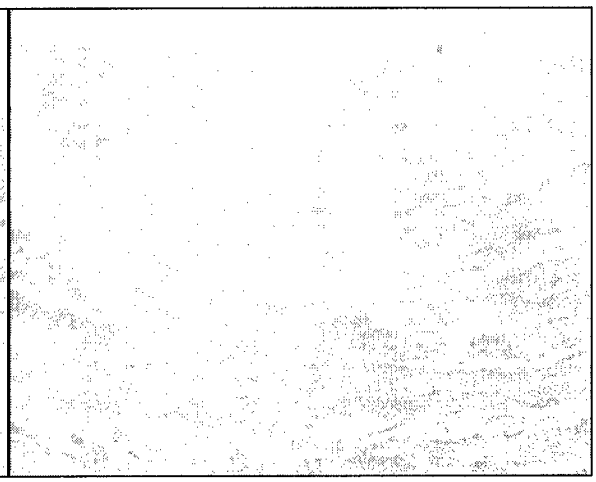


Figure 6) Ethylene Glycol 20X H&E

Mechanical Testing – Statistical Analysis

All mechanical test data were analyzed using a repeated measures ANOVA. This method (similar to a paired t-test) takes advantage of the fact that samples from the same vein graft were treated in different ways for the comparison of those treatment methods. In this way, large variations in the test data that are due to the large variance in donor tissue properties should not hide the effects of the different processing solutions. Statistical significance was considered at $p<0.05$ and a post hoc Neuman-Keuls test was performed where significance was found in the ANOVA.

Circumferential Testing

Circumferential testing simulates the forces that are applied to the walls of the vessel in the circumferential direction when fluid pressure inside the lumen expands the vessel diameter. The testing was conducted in triplicate and the average load at break was used to calculate circumferential tensile strength and the slope of the loading curve was used to calculate a metric of the compliance of the tissue. The raw data from this test are tabulated in Chart 3 (tensile

strength) and Chart 4 (compliance) and the graphs of the ANOVA outputs are in Graphs 2 and 3. Significance in the compliance ANOVA led to a post hoc analysis (Chart 5).

Chart 3) Circumferential tensile strength

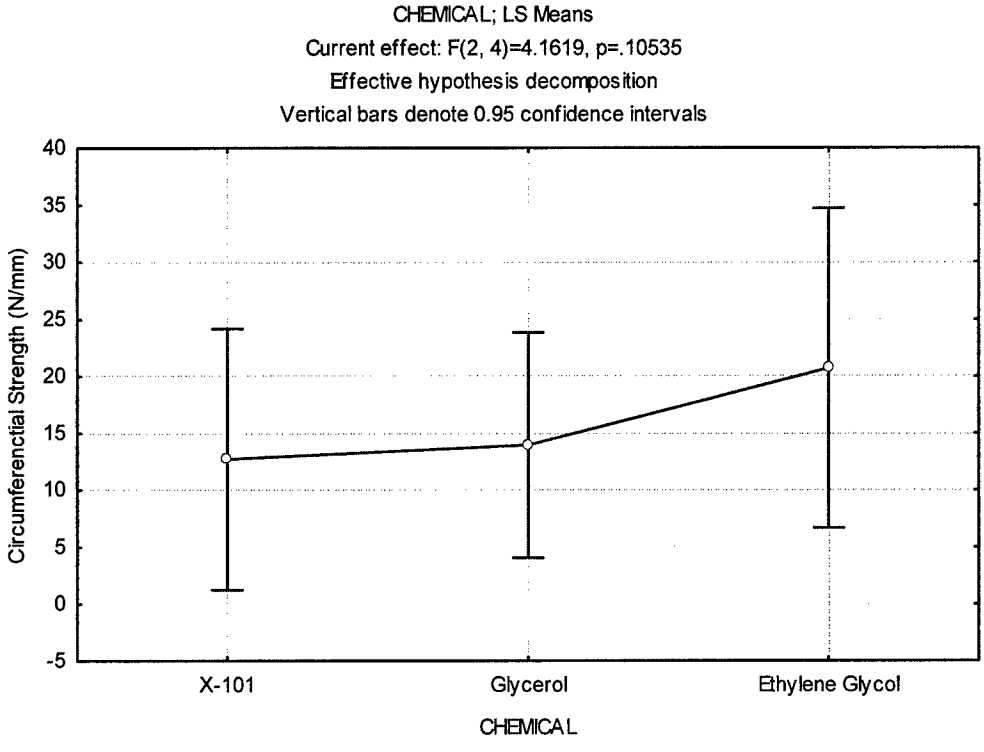
		Load @ Break (N)		Circumferential tensile strength (kN/mm)
Sample ID	Treatment	Average	SD	
13754	X-101	7.52	1.40	3.76E-04
	Glycerol	10.01	2.79	5.01E-04
	Ethylene Glycol	15.86	3.07	7.93E-04
13581	X-101	14.09	3.11	7.05E-04
	Glycerol	12.67	0.39	6.34E-04
	Ethylene Glycol	23.50	1.93	1.17E-03
13755	X-101	17.79	2.85	8.90E-04
	Glycerol	19.05	1.51	9.53E-04
	Ethylene Glycol	24.58	5.08	1.23E-03

Chart 4) Circumferential compliance

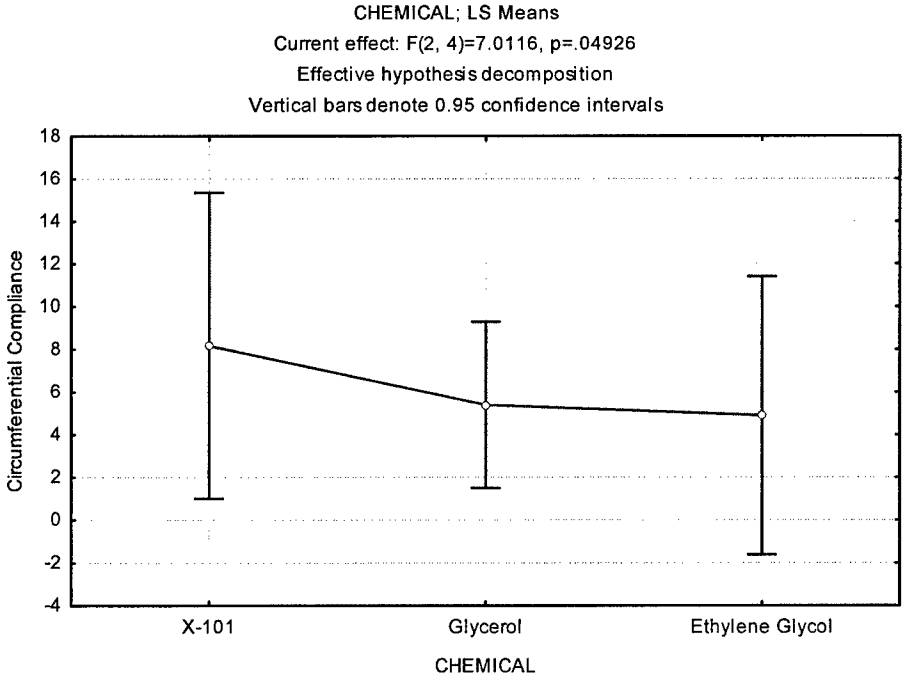
		Compliance (N/%)	
Sample ID	Treatment	Average	SD
13754	X-101	6.77	0.77
	Glycerol	3.66	1.56
	Ethylene Glycol	3.14	2.26
13581	X-101	6.25	1.54
	Glycerol	5.82	1.11
	Ethylene Glycol	3.63	0.04
13755	X-101	11.49	2.85
	Glycerol	6.71	5.37
	Ethylene Glycol	7.90	2.18

Graph 2) Repeated Measures ANOVA of circumferential tensile strength

Appendix – E
In Vitro Evaluation of Different Preservation Methods for the Umbilical Vein Graft



Graph 3) Repeated Measures ANOVA of circumferential compliance



Appendix – E
In Vitro Evaluation of Different Preservation Methods for the Umbilical Vein Graft

Chart 5) Post-Hoc comparison on circumferential compliance:

Newman-Keuls test; variable - circumferential compliance (RR-0033) Approximate Probabilities for Post Hoc Tests Error: Within MS = 1.3336, df = 4.0000

	CHEMICAL	{1}	{2}	{3}
1	X-101		0.042619	0.053962
2	Glycerol	0.042619		0.617348
3	Ethylene Glycol	0.053962	0.617348	

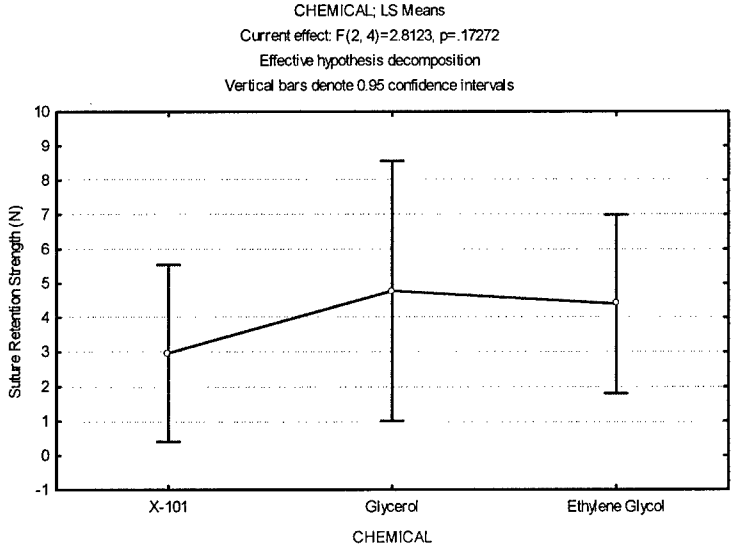
Suture Retention

Suture Retention measures the maximum load (N) it takes for a 5-0 polypropylene suture to tear through the vein graft (Chart 6). Statistically, the maximum load (N) was not differentially affected by the treatments (Graph 4).

Chart 6) Suture retention raw data

Sample ID	Treatment	Maximum Load (N)
13755	Ethylene Glycol	3.71
13755	Glycerol	6.22
13755	X-101	3.95
13581	Ethylene Glycol	5.6
13581	Glycerol	4.92
13581	X-101	3.08
13754	Ethylene Glycol	3.88
13754	Glycerol	3.19
13754	X-101	1.89

Graph 4) Repeated Measures ANOVA of Suture Retention



Appendix – E
In Vitro Evaluation of Different Preservation Methods for the Umbilical Vein Graft

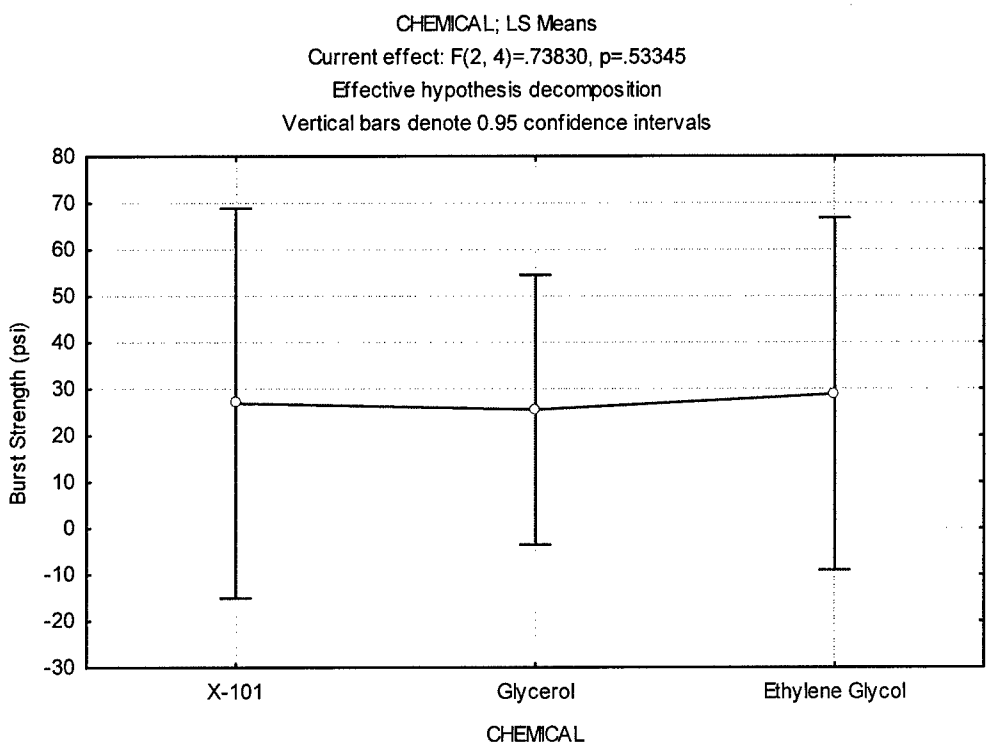
Burst Strength

Burst testing measures the maximum pressure to burst a vein graft. The data are tabulated in Chart 7. Statistically, the burst test data shows no significant difference between preservation solutions (Graph 5).

Chart 7) Burst strength raw data

Cord ID	Treatment	Burst pressure (psi)
13581	X-101	20.8
13581	Glycerol	24.4
13581	Ethylene Glycol	22.4
13754	X-101	13.9
13754	Glycerol	14.4
13754	Ethylene Glycol	17.9
13755	X-101	No data
13755	Glycerol	37.7
13755	Ethylene Glycol	46.3

Graph 5) Repeated Measures ANOVA of Burst strength



4. Discussion

Visual observations and measurable characteristics demonstrate that the different treatments do seem to have varying effects on the tissue; however, tissue to tissue variability was also observed. Even though the three treatments leave the tissue with a slightly different appearance, upon re-hydration they all look the same.

Histologically, X-101, glycerol and ethylene glycol had varying amounts of nuclear debris. Holes, collagen damage, and collagen separation / orientation for all three sample lots and treatments appear similar. Also for all sample lots and treatments the basement membrane and internal elastic lamina was present. Lastly no MHC I or II was detected on any of the slides.

The only mechanical difference detected between the different treatments was the reduced compliance (N/%) in the Glycerol preserved compared with the X-101 frozen tissue. However, there was very high variability in the test results. Repeated measures ANOVAs were performed to avoid the expected lot to lot variation that is apparent in the data. Still, more analysis would be needed to evaluate the true level of detection that these assays are capable of.

5. Conclusions

Based on the comparison criteria of histology, circumferential, suture retention and burst strength testing the data show that the different processing methods are comparable.

Appendix – F
In vivo Evaluation of Glycerol and Ethylene Glycol Preserved Human Umbilical Vein Grafts

INTRODUCTION

A. Purpose:

The objective of the study is to assess the biocompatibility of the test articles relative to the controls. Biocompatibility is indicated by well integrated tissue that is populated by non-inflammatory host cells and supported by neovascularization of the tissue following implant. Based on this criteria, the test articles and control samples were ranked from most biocompatible (a score of 1) to least biocompatible (a score of 4) by the pathologist.

B. Justification:

The laboratory rat is an accepted animal model for microsurgery implantation procedures and biocompatibility testing of biologically derived materials and polymers. Surgical methods used to subcutaneously implant a variety of materials in rats are well described in *Experimental and Surgical Technique in the Rat.*[1] A Medline search of the scientific literature indicates the laboratory rat is an accepted animal model for subchronic testing of both polymeric and biologically derived materials.

EXPERIMENTAL DESIGN

A. General Description:

On Study Day 0 (June 29, 2004), rats were subcutaneously implanted at one of four sites (Figure 1) with one (1) segment from each of two (2) different Test Article umbilical vein graft lots and one (1) segment from each Control Article segment (Table 1). Post surgery weights were recorded twice a week. Rats were observed daily; any abnormalities were recorded.

The study terminated on Study Day 21 (July 20, 2004) all surviving rats were submitted for necropsy. At the time of necropsy surgical implant sites were observed for any abnormalities, implants were removed, photographed, and embedded in paraffin and shipped to Pathology Associates Division of Charles River Laboratories, Worcester, MA, for analysis.

B. Group Assignments:

A total of twelve (12) rats were obtained of which ten (10) were used on study. Two (2) additional rats were received to ensure that only healthy rats were placed on study and to replace any animals that reacted adversely to the surgical procedure.

---

[1] Waynforth and Flecknell, *Experimental and Surgical Technique in the Rat.* 1992.

Appendix – F
In vivo Evaluation of Glycerol and Ethylene Glycol Preserved Human Umbilical Vein Grafts

1) Table 1. Implant Labeling:

Sample	Tissue Type	Storage Conditions
A	Positive control article	-65° to - 90°
B	Negative control article	ambient
C	Test Article – glycerol	4°C ± 1°C
D	Test Article – glycerol	37°C ± 1°C
E	Test Article – ethylene glycol	4°C ± 1°C
F	Test Article – ethylene glycol	37°C ± 1°C

B. Anesthesia and Analgesia:

Charles River Laboratories International provided the anesthesia. Rats were not fasted as stated in the original protocol (Deviation W040707A). Each rat received an intraperitoneal (IP) injection of a ketamine, xylazine and saline mixture in accordance with SOP-2517, *Anesthesia of Laboratory Rodents for Contract Research Services*. When rats were completely anesthetized, surgical implantation of the Test and Control Articles began. When necessary, rats received additional injections of ketamine/xylazine anesthesia in order to complete the implantation procedure. While under anesthesia, the rats were observed for signs of hypothermia and kept warm on heating pads where necessary.

C. Test and Positive Control Article Preparation:

Test Articles and Positive Control Article were supplied to Charles River Laboratories International by Sponsor.

Approximately ninety (90) minutes before implantation the material was aseptically removed from packaging. The Positive Control Article was rehydrated for approximately one (1) hour at room temperature in 0.9% normal saline (two rinses 30 minutes each with 10 mL saline per cm vein). All Test Articles were rehydrated for approximately ninety (90) minutes at room temperature in 0.9% normal saline (three rinses, 30 minutes each rinse, with 10 mL saline per cm vein). These samples were additionally placed on an orbital shaker for the 30 minute rinse cycle.

Each graft (both Test Article and Positive Control) was prepared for implantation by cutting 4 mm rings off of one end of the graft specimen. Prepared implants were stored in labeled sterile Petri-dishes and moistened with saline until needed for implantation.

Samples were taken for pathological analysis from each Test and Control Article. A 0.5 cm section of each type of implant was placed into labeled cassettes (accession number and tissue type) and placed into 10% neutral buffered formalin (NBF). Implants were stored at ambient temperature in fixative until transferred to Pathology Associates Division of Charles River Laboratories International, Worcester, MA. Specimens were shipped by next-day courier on July 7, 2004 with a chain of custody letter.

Appendix – F
In vivo Evaluation of Glycerol and Ethylene Glycol Preserved Human Umbilical Vein Grafts

D. Negative Control Preparation:

Synthetic material was cut into 4 mm rings and soaked in 0.9% normal saline for the same period as outlined above for other implant materials.

E. Test and Control Article Administration:

On Study Day 0, each rat received subcutaneous implants of a 4 mm graft segment at four (4) different sites (Figure 1). Surgery was performed on rats in random order. Two (2) samples were implanted, anteriorly; one (1) right and one (1) left of the spinal midline (right front and left front) and two (2) samples were implanted posteriorly; one (1) right and one (1) left of the spinal midline (right hind and left hind). Each rat received one (1) Negative Control, one (1) Positive Control and two (2) Test Article grafts.

Each type of Test Article appeared only once in any given rat. Both the implant location as well as adjacency of the various articles were randomized so that any possible effect of these parameters on results was minimized. Order of rat implantation was also randomized to nullify effects of experimental drift (Table 2).

Surgery was performed under aseptic conditions. The surgical site of each rat was wiped with Betadyne and alcohol. A small incision was made with scissors at the implantation site. The subcutaneous tissue and fascia were bisected with scissors, ensuring that there was minimal space around each implant, minimal bleeding and no communication between the four (4) implant sites. The pocket was held open while the article was inserted with forceps. Sutures or wound clips were used to close each pocket. The surgical technique was performed upon all implantation sites in the same manner.

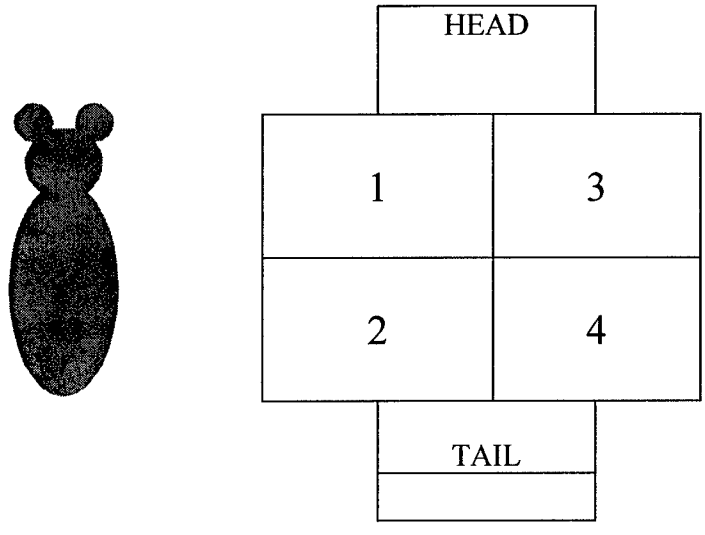
Appendix – F
In vivo Evaluation of Glycerol and Ethylene Glycol Preserved Human Umbilical Vein Grafts

1) Table 2. Implant Schedule:

Rat 1 - #197		Rat 2 - #198		Rat 3 - #190		Rat 4 - #193		Rat 5 - # 195	
A	B	B	A	C	E	F	D	E	A
C	F	F	D	B	A	A	B	B	C

Rat 6 - #185		Rat 7 - #192		Rat 8 - #196		Rat 9 - #191		Rat 10 - #200	
B	E	D	B	A	C	A	F	D	B
D	A	A	F	E	B	B	C	E	A

2) Figure 1. Implant Position Guide:



F. Test and Control Article Histopathology (Study Day 0):

The remaining 0.5 cm Test Article and Positive Control samples were submitted for histopathological analysis. The samples were fixed using the appropriate buffers for subsequent hematoxylin and eosin (H&E), Periodic Acid-Schiff's (PAS) and Verhoeff-Van Gieson (Verhoeff) staining. These samples were sent to the Sponsor for evaluation.

G. Necropsy:

1) Scheduled Necropsy:

Necropsy was carried out at Charles River Laboratories International Health Monitoring (HM) Department, Wilmington, MA. All rats were submitted for necropsy on Study Day 21 (July 20, 2004).

Euthanasia was performed using CO_2 asphyxiation. No gross examinations were performed except to provide brief comments on any inflammation

abnormalities noted at implant sites. Necropsy was limited to implant collection. Implants were collected one at a time from each rat (4 per rat).

A midline dorsal incision through the skin was made, such that skin was reflected back, and implants in the subcutis were visually observed. Abnormalities associated with the implant sites were documented. The Test Article and any associated encapsulating tissue were dissected free from the subcutis.

A digital image of each implant was taken at the time of Test Article collection. Photos included a label with date of necropsy, study number, animal number, and Test Article implant site information (A-D). Implants were placed into labeled cassettes (accession number, animal number, implant site) and placed into 10% neutral buffered formalin (NBF). One fixative container was used per rat. Implants were stored at ambient temperature in fixative until transferred to Pathology Associates Division of Charles River Laboratories International, Worcester, MA.

H. Histology/Histopathology:

All histology and histopathology procedures were performed at Pathology Associates Division of Charles River Laboratories International, Worcester, MA.

The fixed Test and Control Articles and surrounding tissue were appropriately trimmed, embedded in paraffin and several sections obtained that clearly identify the test device/host tissue interface. The resulting slides were stained with hematoxylin and eosin. The samples were read and interpreted by a board-certified veterinary pathologist. The slides were examined by the pathologist in a blinded fashion with no prior knowledge of group assignment for each section. Morphologic parameters that were assessed by light microscopy included, but were not limited to, the following: a) extent of fibrosis/fibrous capsule and inflammation around implant; b) degeneration as determined by changes in tissue morphology; c) number and distribution of the inflammatory cell types, namely polymorphonuclear leucocytes, lymphocytes, plasma cells, eosinophils, macrophages, and/or multinucleated cells; d) presence of necrosis; and e) nature and extent of tissue in-growth into implant (including vascularization).

The pathologist performed a qualitative biocompatibility assessment of the Test Articles and Control Article, including ranked scoring (a score of one (1) representing the most biocompatibility) based upon the following guidelines:

- Less encapsulation is considered more biocompatible
- Less inflammation is considered more biocompatible
- More cell infiltration (non-inflammatory cells) is considered more biocompatible
- Deeper infiltration of host cells (non-inflammatory cells) is considered more biocompatible
- More active vascular channels is considered More biocompatible

Appendix – F
In vivo Evaluation of Glycerol and Ethylene Glycol Preserved Human Umbilical Vein Grafts

OBSERVATIONS AND EXAMINATIONS

A. Clinical Observations:

Observations of each animal were performed and recorded at least once daily for morbidity, mortality and appearance of the surgical sites. Morbidity would include signs of illness such as, but not limited to, emaciation, dehydration, lethargy, hunched posture, unkempt appearance, dyspnea and urine or fecal staining. All findings were recorded in the study notebook on Charles River *Laboratories Daily Observation Form* (FM-279). A clinical observation summary statement may be found in Section X, Part A.1.

B. Animal Weight Data:

Each animal was weighed on Study Day 0. All animals on study were weighed twice weekly for the duration of the study. Animal weights were recorded on Charles River Laboratories *Animal Weight Record Form* (FM-138). A mean animal weight summary statement may be found on Section X, Part A.2.

C. Necropsy Data:

A necropsy summary statement may be found in Section X, Part A.3 of this final report. Copies of IANRs may be found in Section XIII, Appendix, Part C, Attachment 3.

D. Pathology Report:

A pathology summary statement is attached.

STATISTICAL EVALUATION

Statistical manipulations are not required in this study.

RESULTS

A. In-Life Measurements and Observations:

1) Clinical Observations Summary Statement:

At the time of animal receipt, animals were clinically observed and were healthy. No unexpected deaths were observed during the study period. There were no detectable abnormalities prior to scheduled euthanasia on Study Day 21 (July 20, 2004).

2) Mean Animal Weight Summary Statement:

At study initiation, mean animal weight was 244.3 grams (g) with individual animal weights ranging from 180 g to 333 g. During the course of the study, mean animal weights progressively increased in all groups. At study termination, mean animal weight was 272.3 g with individual animal weights ranging from 216 g to 337 g.

A mean weight summary table may be found in Section XI, Part C.1 (Table 3), and a chart of mean animal weight measurements in Section XI, Part C.2 Figure 2).

3) Necropsy Summary Statement:

All live animals on study were submitted to Charles River Laboratories Health Monitoring Department for implant collection. At necropsy, visible implants were detected in all animals surgically implanted with Test Article, with the exception of animal 193 (implant B was not found). Also during the necropsy of animal 198, implant D was cut. Copies of *Individual Animals Necropsy Reports* (IANR; FM-688) may be found in section XIV. Attachment 3.

4) Pathology Summary Statement:

All samples collected at necropsy were sent to PAI for microscopic evaluation. Microscopic findings were graded on a scale from 1-4, (minimal<mild<moderate<marked) according to the intensity and extent of change. A copy of the Pathology Report is attached below.

B. Conclusions:

Overall, rats implanted with Test Article appeared normal throughout the duration of the study and at the scheduled necropsy. No animals died unexpectedly on study.

As expected, mean animal weights increased normally as the study progressed.

In accordance with the attached Pathology Report, the intensities of alterations varied somewhat between each test article as well as between some test articles and the positive control umbilical vein grafts. Test articles stored at 4° C gave similar response to the positive control, while test articles stored at 37° C had less biocompatibility compared to the positive control.

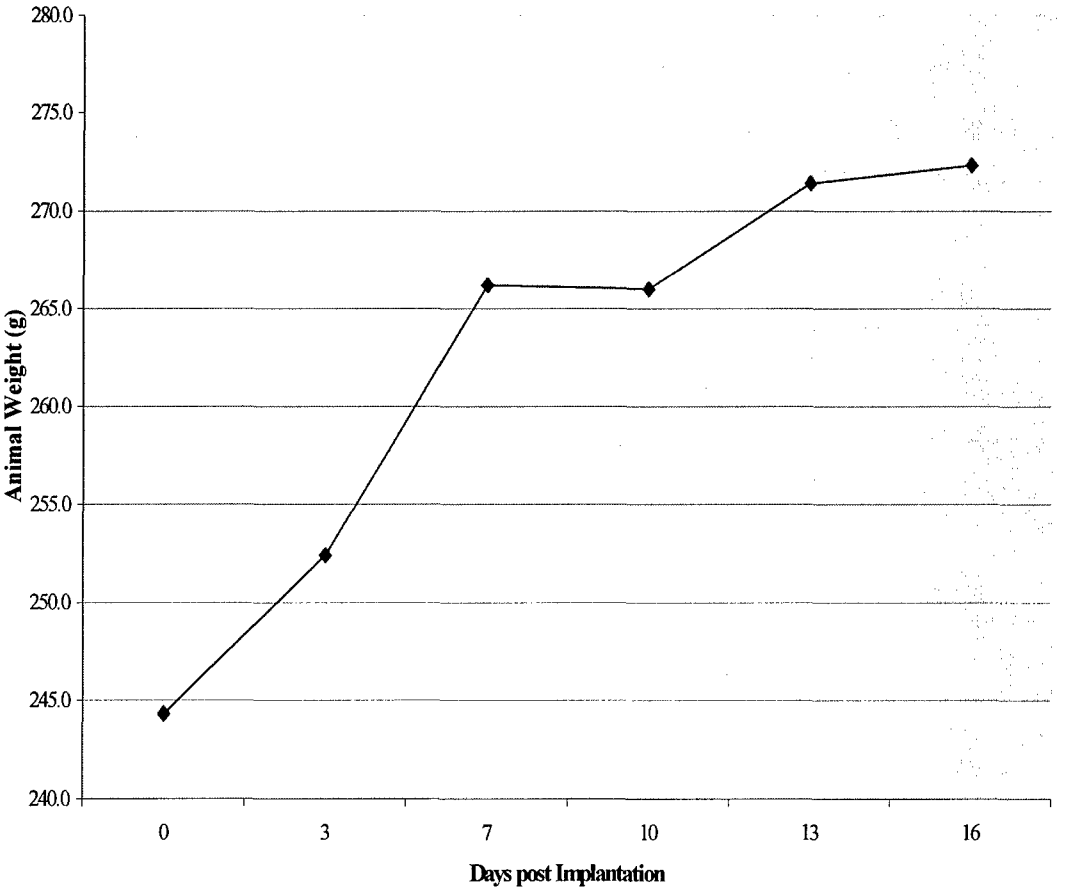
Appendix – F
In vivo Evaluation of Glycerol and Ethylene Glycol Preserved Human Umbilical Vein Grafts

C. Animal Weight Data:

1) Table 3. Mean Animal Weights in Grams ± Standard Deviation (SD):

Group Number	Study Day	0	3	7	10	13	16
	Date	6/29/2004	7/2/2004	7/6/2004	7/9/2004	7/12/2004	7/15/2004
N/A	Mean	244.3	252.4	266.2	266.0	271.4	272.3
	SD	42.8	38.1	35.2	28.2	28.7	33.8

2) Figure 2. Mean Animal Weights in Grams:



Rats were weighed individually twice a week for the duration of the study. Data represented graphically as the average animal weight in grams (g) by group.

CHARLES RIVER
LABORATORIES
Discovery and Development Services
Pathology Associates

DRAFT PATHOLOGY REPORT

Histopathology Services in Support of:

GLP NUDE RAT IMPLANT STUDY

CRL-Wilmington Protocol Number: PR-284-0
CRL-Wilmington Study Number: LFC-0403
PAI STUDY NUMBER: LIFE-0003

SPONSOR:	LifeCell Corp. One Millenium Way Branchburg, NJ 08876
SPONSOR'S REPRESENTATIVES:	Nathaniel Bachrach Telephone: 908-947-1161 Fax: 908-947-1200 Email: nbachrach@lifecell.com Hsin-Chien Tai, Ph.D. Telephone: 908-947-1185 Fax: 908-947-1200
TEST FACILITY:	Research Services Department Charles River Laboratories, Inc., (CRL) 255 Ballardvale Street Wilmington, MA 01887
STUDY DIRECTOR:	Anthony F. Trombino, Ph.D. Telephone: 978-658-6000, Ext. 1471 Fax: 978-988-9093
HISTOPATHOLOGY SERVICES:	Charles River Laboratories Discovery and Development Services Pathology Associates 57 Union Street Worcester, MA 01608

Sponsor: LifeCell Corp.
Subcontractor: Charles River Laboratories DDS, Pathology Associates

PATHOLOGY ASSOCIATES DIVISION DIRECTOR:
Serge Rousselle, D.V.M., D.A.C.V.P.
Telephone: 301-624-2946
Fax: 301-663-8994
Email: srousselle@criver.com

STUDY PATHOLOGIST:
David Garlick, D.V.M., D.A.C.V.P.
Telephone: 508-890-0323
Fax: 508-797-3925
Email: david.garlick@dds.criver.com

1. INTRODUCTION

This pathology report, submitted by Pathology Associates Division, Charles River Laboratories DDS, to LifeCell Corp., represents the pathology findings for the study designated as "GLP Nude Rat Implant Study", LifeCell Corp. Protocol Number PR-284-0, Study Number LFC-0403.

This study consisted of 10 female nude rats which each received subcutaneous implants at four sites, two on each dorsolateral side. The animals were necropsied after 21 days.

2. OBJECTIVES/PURPOSE

The purpose of this study was to evaluate and characterize the relative trends of *in vivo* biocompatibility of human umbilical vein and polymeric implants.

3. STUDY DESIGN

Implant Labeling

Sample	Tissue Type	Storage Conditions
A	Positive Control Article	-65° to -90°
B	Negative Control Article	ambient
C	Test Article - glycerol	4°C ± 1°C
D	Test Article - glycerol	37°C ± 1°C
E	Test Article - ethylene glycol	4°C ± 1°C
F	Test Article - ethylene glycol	37°C ± 1°C

Sponsor: LifeCell Corp.
Subcontractor: Charles River Laboratories DDS, Pathology Associates

3.1. Test and Control Article Administration

On Study Day 0, each rat received subcutaneous implants at four sites, two on each dorsolateral side: an implant of one 4 mm segment from each of two different test article umbilical vein graft lots and one 4 mm segment from each control article segment.

4. ANATOMIC PATHOLOGY

4.1. Euthanasia

On Day 21, animals were euthanized by carbon dioxide asphyxiation.

4.2. Necropsy

Necropsy procedures were performed at Charles River Laboratories Health Monitoring (HM) Department, Wilmington, MA. All animals were subjected to a limited necropsy, which consisted of examination and collection of implant sites only. Implants were fixed in 10% neutral buffered formalin (NBF).

4.3. Histology/Histopathology

All Histology and Histopathology procedures were performed at Pathology Associates Division of Charles River Laboratories, Worcester, MA.

The fixed Test and Control Articles and surrounding tissues were trimmed, embedded, sectioned, and were stained with hematoxylin and eosin, and read and interpreted by a board certified veterinary pathologist. The slides were examined in a blinded fashion with no prior knowledge of group assignment for each section.

Sponsor: LifeCell Corp.
Subcontractor: Charles River Laboratories DDS, Pathology Associates

Microscopic Pathology: Microscopic findings were graded on a scale of 1-4, (minimal<mild<moderate<marked), according to the intensity and extent of change.

For the most part, the host tissue response to the four test articles and the positive control was comparable in morphologic appearance with some variations in the intensity of the observed response to the different implanted umbilical vein grafts. There was usually some variability of the tissue response for each individual specimen, therefore, the grade assigned to each particular morphologic finding was that which was noted for the greater part of the specimen.

A connective tissue capsule surrounded all umbilical vein grafts and intensity was graded as minimal to mild. The capsule when barely perceptible at the edges of the implant was graded as minimal in degree or when up to several fibroblast layers thick around the outer portions of the implant was graded as mild in degree.

Typically, the umbilical vein grafts were infiltrated by fibroblasts through Wharton's jelly and into the wall (media) of the vein. Neovascularization as defined by the presence of new, small caliber vascular channels that also infiltrated the full thickness of the umbilical vein accompanied the fibroblasts. The range of degree for fibroblast infiltration and neovascularization in the umbilical vein graft specimens was mild to moderate.

Mononuclear cells were noted in response to the test articles and the positive control article. Mononuclear cell infiltrates were characterized by the presence of macrophages with negligible numbers of lymphocytes. Granulocytes (neutrophils and eosinophils) were infrequently observed and their intensity/incidence overall was negligible. When mononuclear cell infiltrates tended to be present at the periphery of the grafts this finding was graded as minimal in degree. When inflammatory cells were more readily apparent and also tended to infiltrate into the grafts (within Wharton's Jelly and/or media) in small numbers this finding was graded as mild in degree. When the extent of mononuclear cell infiltration was clearly visible and included locally extensive zones of infiltration this finding was graded as moderate in degree. Moderate numbers of mononuclear cells was noted in only one positive control specimen (Animal No. 195/implant site C).

The epithelial layer of the umbilical vein grafts could not be visualized in any of the umbilical vein graft specimens as the grafts were decellularized prior to implantation. Occasionally, karyorrhectic debris likely originating from necrotic inflammatory cells and/or fibroblasts was observed within the infiltrated portions of the grafts. Cytoplasmic brown pigment was noted in macrophages in many test article implant sites which was likely hemosiderin due to resolving hemorrhage. Some specimens also had microgranulomas centered on hair shaft fragments that were likely ectopically placed during surgical procedures.

The central lumen of the test article and positive control grafts was often occluded by in-growth of fibrovascular tissue, sometimes accompanied by inflammatory cells and pigment-laden macrophages. Fragmentation of the umbilical vein grafts was observed for three specimens: Animal No. 195/implant site A (ethylene glycol 4°C), Animal No. 195/implant site C (positive control graft), and Animal No. 185/implant site C (ethylene glycol 4°C). In a few additional specimens, the umbilical vein grafts were not intact (i.e., fissured) with the observed imperfection extending through the vein and into the vein lumen: Animal No. 192/implant site B (positive control graft); Animal No. 195/implant site A (ethylene glycol 4°C); Animal No. 196/implant site A (positive control graft); and Animal No. 198/implant site D (glycerol 37°C). The cause of this fragmentation and contouring of these few umbilical vein grafts is unknown.

Host tissue response to the negative control article, ePTFE synthetic tubes, was consistent and as expected. Capsular fibrosis around the periphery of the ePTFE tubes was graded as minimal to mild. Mononuclear cell infiltrates, graded primarily as minimal degree, were composed of macrophages and infrequent multinucleated giant cells present at the host tissue-graft interface. Granulocyte infiltrates were negligible. Cellular infiltration (mild to moderate) was noted within the interstices of the graft material primarily consisting of fibroblasts and macrophages while devoid of vascular in-growth. Basophilic deposits (mild to moderate) suggestive of mineral were also present within the device interstices. A fibrovascular stroma (all animals, mild to marked) was also noted within the lumen of the ePTFE grafts, sometimes associated with hemorrhage. Some of the ePTFE specimens also had hair shaft-associated microgranulomas.

Sponsor: LifeCell Corp.
Subcontractor: Charles River Laboratories DDS, Pathology Associates

In an attempt to rank specimens according to a biocompatibility score (per protocol Amendment 1), the following guidelines were employed to help rank the tissue response seen to each implant material:

- Less **encapsulation** is more biocompatible
- Less **inflammation** is more biocompatible
- More/deeper **cellular infiltration** (non-inflammatory) is more biocompatible
- More **vascular channels** is more biocompatible

Accordingly, each of the above categories was ranked on a scale of 1 (most biocompatible) to 4 (least biocompatible) as per Protocol Amendment II. The scoring was based on trends for microscopic incidence and intensity of the finding for each implant (see Pathology Table Number 1 for tabular summary of findings) as follows: 1 = most biocompatible, 2 = more biocompatible, 3 = less biocompatible, 4 = least biocompatible. The biocompatibility score for each implant type is presented below in Pathology Text Table 1. For each implant type the numbers assigned to each of the four categories was totaled and then divided by 4 to give an average biocompatibility score.

Pathology Text Table 1
Biocompatibility Scores

Implant Type	Fibroblast In-growth	Vascular In-growth	Inflammation	Capsule	Total Score	Average Score
Glycerol 4°C	2	2	2	1	7	1.75
Glycerol 37°C	3	3	1	1	8	2.0
Ethylene Glycol 4°C	2	2	2	1	7	1.75
Ethylene Glycol 37°C	3	3	1	2	9	2.25
Positive control article	2	2	2	1	7	1.75
Negative control article	3	4	1	2	10	2.5

According to this subjective schema, the umbilical vein graft glycerol 4°C and umbilical vein graft ethylene glycol 4°C were deemed to have the lowest biocompatibility scores among the test articles as well as comparable biocompatibility scores to the positive control umbilical vein graft. Additionally, these same two test article umbilical vein grafts stored at 4°C were deemed to be slightly more biocompatible than umbilical vein graft glycerol at 37°C and umbilical vein graft ethylene glycol at 37°C. All test article umbilical vein grafts appeared to be somewhat more biocompatible than the negative control article, ePTFE synthetic tubes.

Conclusions

In general, the morphologic appearance of the host tissue response to the four test article umbilical vein grafts was similar to the positive control human umbilical vein grafts. Umbilical vein grafts were encapsulated, infiltrated by fibroblasts and new blood vessels, and associated with mononuclear cell infiltrates. However, the intensity of these alterations varied somewhat between each test article as well as between some test articles and the positive control umbilical vein grafts.

In an attempt to further define tissue biocompatibility for each test article as compared to the controls, a scoring system was employed to further assess relative biocompatibility.

Accordingly, the umbilical vein graft glycerol 4°C and umbilical vein graft ethylene glycol 4°C were deemed to have the lowest biocompatibility scores among the test articles as well as comparable biocompatibility scores to the positive control umbilical vein graft. Additionally, the glycerol 4°C and ethylene glycol 4°C grafts appeared to be slightly more biocompatible than umbilical vein graft glycerol 37°C and umbilical vein graft ethylene glycol 37°C. However, it should be realized that the grading scheme employed was subjective, i.e., biocompatibility score assignment was based on interpretation of qualitative data.

Trends regarding the type and/or intensity of host tissue response to the different umbilical vein grafts implants were noted as follows:

- Comparing test article umbilical vein grafts, there tended to be an increase in the degree of tissue in-growth (fibroblasts/neovascularization) into the grafts stored at 4°C as compared to the grafts stored at 37°C.

- Comparing test article umbilical vein grafts, there tended to be a lower intensity of mononuclear cell infiltrates associated with the grafts stored at 37°C as compared to the 4°C grafts.

- Comparing test article umbilical vein grafts with the positive control umbilical vein grafts, there was tendency to comparable intensity of tissue in-growth between the control grafts and the grafts stored at 4°C.

- For all umbilical vein grafts, the degree of inflammation tended to parallel the degree of tissue in-growth.

Pathology Signature

David S. Garlick, D.V.M., / Date
Diplomate, A.C.V.P.
Study Pathologist

Microscopic Data Table

Histopathology Report
GLP NUDE RAT IMPLANT STUDY
PAI/Worcester Study Number: LifeCell-0003
Sponsor: LifeCell Corp.
CRL/Wilmington Study Number: LFC-0403

Pathology Table 1

Tissue/Finding Animal #	185	190	191	192	193	195	196	197	198	200
Positive Control Article					*					
Fibroblast infiltration, full thickness	3	2	2	3		3	3	3	3	2
Neovascularization, full thickness	3	2	2	3		3	3	3	3	2
Fibrous Capsule	1	2	1	1		2	1	2	2	1
Mononuclear cell infiltrates	2	2	1	2		3	2	2	2	1
Granulocyte infiltrates							1			
Lumen occlusion, umbilical vein	P	P		P				P	P	P
Microgranuloma	1					1			2	
Lumen not visualized			P			P				
Cytoplasmic pigment, macrophage		1								1
Graft fragmentation						P				
Lumen fissure				P			P			
Negative Control Article										
Fibrous Capsule	1	2	1	2	2	2	2	2	2	2
Mononuclear cell infiltrates	1	1	1	1	1	1	2	1	2	1
Granulocyte infiltrates									1	
Microgranuloma		1			1		1	2	2	
Cytoplasmic pigment, macrophage	1				2	1	2		2	1
Fibrovascular stroma, inner device surface	3	2	2	3	3	4	4	4	4	4
Hemorrhage, inner device surface	1				2	2	3		2	2
Basophilic deposits, device interstices	2	2	2	3	2	3	2	2	3	2
Cellular infiltration, device interstices	2	3	2	3	2	2	3	3	2	2
Test Article - Glycerol 4° C										
Fibroblast infiltration, full thickness		3	2			3	3	2		
Neovascularization, full thickness		3	2			3	3	2		
Fibrous Capsule		1	1			1	1	2		
Mononuclear cell infiltrates		2	1			2	2	1		
Lumen occlusion, umbilical vein		P	P			P	P	P		
Microgranuloma		1	1			2				
Cytoplasmic pigment, macrophage		1	1			1	1	1		
Test Article - Glycerol 37° C										
Fibroblast infiltration, full thickness	2			2	2				2	2
Neovascularization, full thickness	2			2	2				2	2
Fibrous Capsule	1			1	1				2	1
Mononuclear cell infiltrates	1			1	1				1	1
Lumen occlusion, umbilical vein					P					P
Microgranuloma	1			1	1				1	
Cytoplasmic pigment, macrophage					1					1
Lumen fissure									P	
Test Article - Ethylene Glycol 4° C										
Fibroblast infiltration, full thickness	3	3				3	2			3
Neovascularization, full thickness	3	3				3	2			3
Fibrous Capsule	1	1				2	2			1
Mononuclear cell infiltrates	2	2				2	2			1
Lumen occlusion, umbilical vein		P					P			
Microgranuloma	2	1					2			1
Cytoplasmic pigment, macrophage							1			
Graft fragmentation	P					P				
Lumen fissure						P				
Test Article - Ethylene Glycol 37° C										
Fibroblast infiltration, full thickness			2	2	2			2	2	
Neovascularization, full thickness			2	2	2			2	2	
Fibrous Capsule			1	1	2			2	2	
Mononuclear cell infiltrates			1	1	1			1	1	
Lumen occlusion, umbilical vein				P	P			P	P	
Microgranuloma				1					1	
Cytoplasmic pigment, macrophage					1			1		

All findings were graded subjectively using a 4-point scale; 1=minimal, 2=mild, 3=moderate, 4=marked; P=present.
* Sample not submitted

Appendix – G
Stability of Glycerol Preserved Human Umbilical Vein Grafts

1.0 Objectives

To determine the stability of umbilical vein grafts (UVG) preserved in glycerol at either 4°C or 20°C after ~1 and 3 months. The stability criteria included sterility, histology, and mechanical properties.

2.0 Materials/Equipment

2.1 0.9% Sodium Chloride solution (saline) (part # 112P0050)
2.2 Glycerol USP grade (Sigma) (part # 112P0022)
2.3 Refrigerator (equipment # 1537)
2.4 Low temperature incubator (equipment # 1508) set to 20°C
2.5 60mL Nalgene bottles
2.6 50mL conical tubes
2.7 Aluminum foil
2.8 7" X 11" foil bags

3.0 Methods

3.1 Three cords were aseptically dissected and processed through the 3rd PBS wash after the DNase step. The tissue was then subjected to the glycerol treatment as follows:

(Note: glycerol solution on %v/v basis with saline.)

- 10mL/cm at room temperature shaking at 85 rpms.
- Incubation with 40% for 1 hour ± 15 minutes.
- Decant the 40% and incubate with 55% for 1 hour ± 15 minutes.
- Decant the 55% and incubate with 70% for 1 hour ± 15 minutes.
- Decant the 70% and incubate with 85% for 1 hour ± 15 minutes.

Samples were packaged with sufficient 85% to cover the sample and placed in either 60mL bottles or 50mL conical tubes depending on the experiment. Samples were shielded from light using foil bags or aluminum foil. Samples were labeled with protocol number, sample ID, storage temperature, and storage time duration.

4.0 Deviations

4.1 Suture retention testing was conducted at a rate of 50mm/min instead of 150mm/min for the 3 month time point (experiment 2), due to operator error. Experiment 238-33, in notebook 238 pages 68-69, was set up to examine the effect between running a suture retention test at a rate of 50mm/min versus 150mm/min. Two vein grafts processed as per SOP, were cut in half and half of each was tested at each speed. The max load (N) strength values were compared using a T-test ($p<0.05$) and even though the 2 speeds did not show significant difference, the samples subjected to the 50mm/min rate were ~23% lower than the 150mm/min samples. The data from the 3 months time point should be considered in light of this finding.

5.0 Results and Analysis

5.1 Data Analysis

Data were analyzed using a repeated measures ANOVA ($p<0.05$).

- Effect of temperatures.

- Effect of time.
- Interaction effect of temperature and time.

Where possible, the data were also compared to the frozen umbilical vein graft process.

5.1.1 Circumferential testing

Circumferential testing simulates the forces that are applied to the walls of the vessel in the circumferential direction when fluid pressure inside the lumen expands the vessel diameter. The data evaluated from this testing includes load at break (N) and compliance (N/%) (Chart 1).

Chart 1) Circumferential testing data

		Temperature					
		4°C			20°C		
Test	Sample ID	T = 0	T = 1	T = 3	T = 0	T = 1	T = 3
Compliance (N/%)	10608	2.76	9.11	5.18	6.64	6.99	4.26
	13722	4.32	5.70	2.37	9.56	15.09	2.70
	SFH-8	11.92	13.29	6.48	10.57	4.24	6.38
	Frozen	No data available					
Load at break (N)	10608	10.99	18.31	19.70	7.06	9.37	8.88
	13722	11.83	6.83	8.46	17.50	16.80	10.73
	SFH-8	14.12	21.93	22.30	21.79	19.96	19.64
	Frozen	No data available					

Samples from 1 and 3 months and either 4°C or 20°C are comparable to time zero at either temperature for circumferential compliance (N/%) and circumferential load at break (N).
No comparison can be made to the frozen umbilical vein graft as no data were submitted within the IDE.

5.1.2 Multi-puncture testing

Multi-puncture testing uses the same forces as the circumferential test, only the sample is subjected to 24 punctures from a 16 gauge needle. This is to simulate ~18 months of clinical use. The data evaluated here is the load at break (N/cm) (Chart 2).

Chart 2) Multi-Puncture testing data

		Temperature					
		4°C			20°C		
Test	Sample ID	T = 0	T = 1	T = 3	T = 0	T = 1	T = 3
Load at break (N/cm)	10608	9.11	14.76	3.93	7.07	5.90	2.05
	13722	6.82	5.71	4.60	11.19	6.19	8.34
	SFH-8	10.29	14.02	14.11	11.28	12.64	6.32
	Frozen	5.7 ± 1.3 (n = 10)					

After three months time, samples were comparable for both temperature and time. However, sample 10608 was lower than data from the frozen design for both temperatures at the 3 month time point.

5.1.3 Suture retention strength testing

Suture retention strength measures the maximum load (N) required to pull a 5-0 polypropylene suture through the vein wall. The acceptance criteria for the ability of a vein graft to resist suture tear must be greater than 1N. In addition to acceptance criteria, the data will be used for tracking purposes and comparison over time (Chart 3).

Chart 3) Suture retention strength data

		Temperature					
		4°C			20°C		
Test	Sample ID	T = 0	T = 1	T = 3	T = 0	T = 1	T = 3
Maximum load (N)	10608	6.84	5.29	2.70	9.16	6.53	3.60
	13722	5.30	7.49	1.61	5.04	3.54	2.41
	SFH-8	4.36	3.08	3.00	6.50	4.45	3.92
	Frozen	> 1					

Samples from 1 month and either 4°C or 20°C were comparable to time zero at either temperature. In contrast, the 3 month samples decline significantly in value compared to both time zero and 1 month samples. This decrease may either be a storage effect and/or partially attributable to the deviation in the test method (see deviation 6.1) Even though it is unknown which factor may have had a greater influence in the decline of the 3 month time points, all values are greater than 1N and meet the acceptance criteria for this test.

5.1.4 Burst strength testing

Burst strength testing measures the pressure required to cause an acute burst of the vein graft. The acute burst pressure must be above 400mmHg to be acceptable (Chart 4).

Appendix – G
Stability of Glycerol Preserved Human Umbilical Vein Grafts

Chart 4) Burst strength data

		Temperature					
		4°C			20°C		
Test	Sample ID	T = 0	T = 1	T = 3	T = 0	T = 1	T = 3
Burst strength (mmHg)*	14545				736	1264	1184
	14544				803	1035	1143
	14594				1715	2296	1215
	14587	1858	2021	2639			
	14408	1827	1843	1934			
	14610	939	1569	1934			
	14152	827	1293		652	543	
	SFH-9	1143	1551		1676	1448	
	10577	569	672		564	776	
	Frozen	> 400					

* Data for the test is recorded in pounds per square inch (psi) and converted to mmHg by multiplying the psi value by 51.715.

All values above met the acceptance criteria. Over time there was no effect on the burst pressures at either temperature. (Note: In this experiment, temperature and time could not be evaluated within the same analysis due to the experimental design).

5.1.5 Histological data

Histology was used to evaluate the microscopic characteristics of the tissue. The tissue was subjectively scored on a scale of 1-4, with 1 being the best, in the following categories.

- Holes
- Collagen Damage
- Collagen separation / orientation

In addition to these characteristics, samples were viewed for significant remaining nuclear material and the presence of a basement membrane and internal elastic lamina (Chart 5).

Appendix – G
Stability of Glycerol Preserved Human Umbilical Vein Grafts

Chart 5) Histology scores

		Temperature					
		4°C			20°C		
	Sample ID	T = 0	T = 1	T = 3	T = 0	T = 1	T = 3
Nuclear Debris	SFH-18B	Significant	Significant	Significant	Significant	Significant	Significant
	SFH-107	Significant	Not Significant	Significant	Significant	Significant	Significant
	SFH-160	Significant	Significant	Significant	Significant	Significant	Not Significant
Holes	SFH-18B	2.0	1.5	2.5	2.0	3.0	2.5
	SFH-107	1.5	1.5	1.5	1.5	1.5	1.5
	SFH-160	1.5	1.5	2.5	1.5	2.5	2.0
Collagen damage	SFH-18B	2.0	2.0	2.0	2.0	3.0	3.0
	SFH-107	1.5	1.5	2.0	2.0	2.5	3.0
	SFH-160	1.5	3.0	3.0	3.0	3.0	3.0
Collagen separation / orientation	SFH-18B	2.5	2.5	2.0	2.5	2.5	3.5
	SFH-107	1.5	2.0	2.0	1.5	2.0	2.5
	SFH-160	2.5	3.0	3.0	3.0	3.5	3.0
Basement membrane	ALL	Present					
Elastic Lamina	ALL	Present					

NOTE: Scoring was performed by Senior Histologist.

The subjective nature of the histology scoring system, limited the data analysis to qualitative descriptions only. In most cases there was still a significant amount of nuclear debris and in all cases the basement membrane and internal elastic lamina were present. The holes, collagen damage and collagen separation / orientation scores increased from time zero. Also the 4°C samples scored better when compared to the 20°C samples.

5.1.6 Microbiological data

Viromed laboratories conducted standard sterility testing on 1cm segments of tissue using a minimum 600ml media. No growth was the only acceptable result for this test (Chart 6).

Chart 6) Microbiology data

	Temperature	4°C			20°C		
	Sample ID	T = 0	T = 1	T = 3	T = 0	T = 1	T = 3
Microbiology	SFH-18B	No growth	No growth	Growth (1)	No growth	No growth	Growth (2)
	SFH-107	No growth	No growth	No growth	No growth	No growth	No growth
	SFH-160	No growth	No growth	Growth (3)	No growth	No growth	No growth

1) Coagulase Negative Staphlococcus
2) Propionibacterium Acnes
3) Bacillus Sp. Not Anthracis

The data implies that over time organisms are growing in the tissue; however, this might not be the case. There may be a distribution of organisms along the length of the graft that could account for some

samples containing micro-organisms while others will not. In addition, the no growth requirement on the sterility testing was for samples processed in a controlled clean room environment as opposed to a standard laboratory area that the samples were actually processed in.

6.0 Conclusions

6.1 Umbilical vein grafts (UVG) preserved in glycerol stored at either 4°C or 20°C after ~1 and 3 months are comparable to the same UVG stored for 0-2 weeks for the following criteria:

- Circumferential load at break (N)
- Circumferential compliance (N/%)
- Multi-puncture load at break (N)
- Burst strength

6.2 For Suture strength the data collected on UVGs preserved in glycerol stored at either 4°C or 20°C after ~3 months were significantly different from the same UVG stored for 0-2 weeks. However, no conclusions can be drawn due to the deviation in applied loading rate

6.3 No conclusions will be drawn at this time from the histology data because of its subjective nature.

6.4 No conclusions will be drawn at this time from the microbiology data because of the difference in processing environments.

6.5 Based on the data, umbilical vein grafts stored in 85% v/v glycerol solution may be stored at either 4°C or 20°C for up to 3 months.

Appendix - H
Evaluation of Human Umbilical Vein Graft as an AV Shunt in a Primate Model

Objectives

The human umbilical vein graft is intended to function as an arteriovenous (AV) access graft in hemodialysis patients. The only large animal study performed to date was a porcine model[1] where the results included severe inflammatory response attributed to cross-species incompatibility. Cross-species immune response can play a role in chronic graft failure so no further chronic studies were pursued. Recent data in a hernia repair model using AlloDerm in the African Green Monkey indicate that there may be close enough homology with humans for processed human tissue to avoid significant immune reactivity.

The objective of this study was to evaluate the suitability of the African Green Monkey for animal implantation with the Human Umbilical Vein Graft for the purpose of evaluating the safety of that graft.

Using the longest graft segment that can be achieved without compromising the graft or animal, the Human Umbilical Vein Grafts were implanted in large male vervets (one per animal) as an AV shunt from the Iliac/femoral artery to the femoral vein. Needle access was planned as a possible endpoint to demonstrate the potential for a chronic multi-stick model. Grafts were implanted for 2 and 6 weeks, three for each time point, with 3 additional grafts included with an unplanned duration. Synthetic control grafts were also implanted (one graft for each time point). Serum was obtained prior to and during the study to evaluate the immune response of the animal to the graft.

Results

A. Study observations

Twelve male African Green monkeys (Vervet, *Cercopithecus aethiops*) were identified by number and randomly assigned to either study or control group. All animals were screened for general health and quarantined for 2 weeks before study entry. All monkeys were housed in individual cages and allowed free access to water, fruits, and monkey food. At the time of surgery the animal weights ranged from 4.45 - 5.62 kg.

Umbilical Vein Grafts (UVG) were transported to the animal testing facility on dry ice. Temperature of the grafts was maintained below -30°C during transport. After transport the grafts were maintained on dry ice until within 4 hours of implantation. The standard thaw and rehydration procedure described in the DFU was used before implantation.

- Test Articles

  Human Umbilical Vein Grafts that were processed per LOP09.01.058 and maintained at -65°C to -90°C until shipment on dry ice to the test site. Test graft articles consisted of approximately 6-8 cm long pieces of whole grafts. The test grafts used in this study were lot numbers U00023, U00033, U00037B.

- Control Articles

[1] LCP-2002-10-02 – performed in 2002.

Appendix - H
Evaluation of Human Umbilical Vein Graft as an AV Shunt in a Primate Model

Segments of 6-8 cm × 6 mm diameter ribbed ePTFE implanted in a similar manner to the test article in 3 additional animals (one for each time-point).

An anatomical comparison of the upper leg / groin area to the neck of the animals indicated that the former was a more likely candidate for successful grafting. Monkeys were anesthetized per the protocol and the iliac artery and femoral vein were exposed by sharp and blunt dissection. A segment (6-8cm) of umbilical vein graft or synthetic (ribbed) ePTFE graft (6mm diameter) was anastomosed to the iliac artery and femoral vein creating an AV shunt. Non-absorbable 6-0 or 7-0 sutures were used. The loop of graft was placed subcutaneously to allow non-invasive evaluation of flow and cannulation. Appropriate antibiotics were administered for 3 days after surgery. No antiplatelet or anticoagulant agents were given during or following surgery.

B. Implantability

Nine UVG segments from three different UVG and three synthetic grafts were successfully implanted - one in each of 12 animals – by Dr. Shiji Qi, over a three day period. One control graft and three UVGs from a single lot were implanted each day. See Table 1 for additional implantation details. Patency was verified by palpation and/or Doppler flow probe (Appendix A – Implant Data Forms).

The grafts were evaluated for patency immediately after completion of implantation and then again after closure by palpation of the graft. An available Doppler flow probe (Smart Doppler ES-1000SPM, Koven Technologies, St. Louis MO) was used to evaluate its ability to measure flow velocities in the implanted grafts. While there is no certainty regarding the quantitative ability of the probe, it was found to qualitatively verify patency of the grafts.

There were no cases of suture pull-out or significant bleeding during implantation. In one case the UVG twisted when arterial flow was initiated in the graft causing a tear in the femoral vein tissue near the anastomosis that was successfully repaired. The synthetic grafts had minor bleeding at the suture holes that was easily stopped with pressure. Despite four grafts with 2 or more turns or coils (#O5481, #O5486, #O5496, #O5493) and a graft loop with a radius of approximately 0.5 cm for the UVG, there were no instances of kinking. The ribbing on the synthetic graft provided protection from kinking by limiting the radius of the graft loop. Photographic records of the implanted grafts *in situ* and after surgical closure are included as Appendix B.

Table 1: General Imlant Data (Appendix A – Implant Data Forms)

Animal Number	Implant Number / Type	Animal Weight (kg)	Date of implant	Mean Flow (cm/s)	Procedure time (min)	# coils
O5480	U00033	4.87	4/20/2004	4.5	67	0
O5481	U00023	4.51	4/19/2004	2.4	80	2
O5485	U00037B	4.76	4/21/2004	3.2	50	1
O5486	U00023	4.76	4/19/2004	4.1	90	3
O5496	U00037B	5.19	4/21/2004	5	50	2
O5499	ePTFE control graft	5.34	4/19/2004	2.8	110	N/A

O5109	U00037B	4.79	4/21/2004	3.5	not recorded	1
O5484	U00023	5.59	4/19/2004	5	60	not recorded
O5460	ePTFE control graft	5.62	4/20/2004	2	76	N/A
O5494	U00033	4.63	4/20/2004	5.1	65	1.5
O5483	ePTFE control graft	4.98	4/21/2004	3.5	85	N/A
O5493	U00033	4.45	4/20/2004	11.2	60	2

C. Deviations

Storage temperature: The protocol calls for storage temperature of the grafts to be maintained at -65°C to -90°C. During shipment of the grafts on dry ice the temperature probes within the shipper briefly reached as high as -30°C[2]. The actual temperature of the grafts at that time is unknown. This deviation in storage is not expected to influence the results of this study. Sections of the grafts obtained at the time of implantation (and after shipment) were evaluated histologically and were not found to be any different than pre-shipment sections.

Control grafts: The protocol called for an 8 mm diameter crimped Bionit Dacron graft (C.R. Bard, Inc., Billerica MA). A 6mm ePTFE graft was used instead. This graft material is the standard of care in the hemodialysis industry and therefore a more appropriate control for the UVG in this study. The deviation does not affect the results of the study.

Early sacrifices: One umbilical vein graft was disturbed by the monkey after five weeks but clotted off before resulting in death of the animal and two other grafts (one control and one UVG) had been repeatedly exposed by the monkey and secondary and tertiary closure of the surgical site was not healing. In these cases the monkeys were sacrificed at five weeks rather than six weeks to avoid the risk of losing additional animals from the study. The deviation impacts the data as it allows for an evaluation of the chronic response of five weeks duration instead of six weeks.

Data completion: The protocol calls for evaluation of cytokine levels and complement activation if there is a sign of inflammation. The immunological assessment provided by the evaluation of antibody levels and immunohistochemistry were later considered to be sufficient information to assess the model.

D. Adverse Events

All adverse events have been defined and reported in an adverse event report from the Staff Veterinarian at the Behavioral Sciences Foundation (Appendix C). Adverse events in this study included ischemia, graft rupture and animal death. A degree of ischemia of the lower extremity was caused by diversion of the arterial flow. The ischemia was noted as a qualitative temperature difference between implanted and contralateral limbs and in some cases temporary immobility of the foot on the implanted limb. Three animals died during the course of the experiment. The cause of death in those cases was linked to exposure and disruption of the graft by the animal. In another case the graft was torn open by the animal but clotted off by itself. This event precipitated an early sacrifice of the animal at five weeks. Full description of these events and photographic

[2] experiment 238-03 "International shipping package containing vein grafts U00023, U00033, and U00037B"

record are included in the Adverse Event report (Appendix C). Another animal intended for sacrifice at six weeks was sacrificed at five weeks due to uncertainty surrounding the likelihood of the animal disrupting the graft.

E. Patency

Patency was evaluated on a weekly basis non-invasively by palpation of the graft and by Doppler ultrasound. After sacrifice, the grafts were explanted and flushed with normal saline or RPMI solution to remove non-adherent blood. This procedure also served to further verify patency. All grafts in planned sacrifices, UVG and synthetic, remained patent (Table 2).

Explanted grafts were sent to LifeCell for dissection and preparation for histological sectioning and staining. The photographic record of the explant dissection demonstrates little adhesion of blood or thrombus to the lumen of the UVG (Appendix D).

Table 2: Explant duration, patency record and time to hemostasis after cannulation.

Animal #	Graft	Duration (days)	Duration Group	Patent at Explant	Time to hemostasis (min)
05499	ePTFE	16	2 week	Yes	not provided
05486	U00023	16	2 week	Yes	5
05480	U00033	15	2 week	Yes	>5
05496	U00037B	14	2 week	Yes	3
05460	ePTFE	36	5-6 week	Yes	2
05483	ePTFE	42	5-6 week	Yes	3-4
05484	U00023	36	5-6 week	Yes	3-4
05494	U00033	36	5-6 week	Yes	3
05493	U00033	43	5-6 week	Yes	5-6
05481	U00023	14			
05485	U00037B	10	No data colleted due to graft disturbance and subsequent animal death		
05109	U00037B	17			

F. Puncturability / Hemostasis

Prior to explantation the grafts were cannulated using a 16g needle as prescribed in the protocol addendum. Hemostasis was achieved using external pressure in <5 minutes in four of the 6 cannulated grafts with the other two requiring >5minutes. One synthetic graft (#O5499) was reported to have uncontrolled bleeding (Table 2). In two cases high pressure was reported during the cannulation procedure indicating a restriction in venous outflow likely the result of the diameter mismatch from the graft (6-8mm) to the vein (2-3mm).

G. Animal Observations – photographic record

Appendix - H
Evaluation of Human Umbilical Vein Graft as an AV Shunt in a Primate Model

H. Explant Record – gross evaluation

The graft were photographed at explant and shipped to LifeCell Corporation in RPMI on ice. Upon receipt of the tissue the grafts were photographed, dissected and sectioned into the following pieces:

- Arterial anastamosis was bisected ½ for sucrose cryoprotectant (OCT) and ½ for formalin storage.
- Serial sections (2 cm) of the graft were made starting from the arterial side after the anastomosis. They were alternately stored in OCT and formalin and numbered from one and increasing from arterial end to venous end.
- Venous anastomosis was bisected ½ for OCT and ½ for formalin storage.

Photographs were taken of the lumen of the graft and the anastomoses (Appendix D).

I. Biological Response

Histological evaluation

An independent histopathological review of hemotoxylin and eosin (H&E) stained sections was performed on all graft sections for two different groups of explants: 1.) those explanted at 14 days (including all grafts explanted from non-surviving animals) and, 2.) those explanted at 5-6 week durations. The final summary report is attached as Appendix E. This report indicates that at early time points (14 day) there was a significant mixed inflammatory response to the graft consisting of polymorphonuclear cells (primarily neutrophils) and macrophages (with a minor component of giant cells). At 5-6 weeks, a mixed inflammatory response was still present but notably reduced. Also, by 5-6 weeks, there was evidence of healing (resolution of the inflammation) with concomitant new matrix (fibrous connective tissue) formation. There was an absence of aneurysm, tissue dissection, or significant thrombus formation. The early inflammatory response and histology are typical for biological implants.

Immunologic response

The cellular response described by H&E pathology was evaluated further using immnohistochemistry. The sections were stained for the presence of macrophages (CD68), T cells (CD3) and B cells (CD20). There was extensive infiltration of macrophages at 14 days especially around the sutures and within the graft as well. This is an expected response to a foreign (not yet integrated) material and is generally understood as the necessary precursor to an infiltration of host cells in a subsequent healing response. The degree of macrophage infiltration was milder and more localized at five and six weeks indicating an acceptance of the tissue. In contrast, macrophages were found in all sections in the host tissue surrounding the synthetic grafts. T cells were found in the host tissue surrounding the graft. However, occasional T cells were found in the wall of vein grafts, especially at the early time points. The explants from two monkeys at five and 6 weeks (#05494 & #05493) showed minimal infiltration of T cells. B cells were generally not observed in most of the sections. In some cases B cells were found in the monkey tissue surrounding the grafts.

Serum was obtained before implantation and at regular intervals following implantation. The serum was stored refrigerated and shipped frozen to LifeCell Corporation after completion of the study. The serum was tested to determine if the animals raised antibodies to the implanted grafts or equivalently processed grafts from different donors. Overall, vascular grafts induced an IgG response in monkeys 14 days post-implant although IgM antibodies remained similar to the pre-implant levels. It is unlikely that monkeys respond to donor specific antigen epitopes since less binding to donor-specific grafts than to generic grafts was detected (Appendix F - Immunology

Summary Report). This data indicates that the tissue has been effectively decellularized as defined by the removal of antigenic capacity.

Model Assessment

There are a number of aspects of this model that require further development and/or mitigation and may affect the ability to use this model for chronic evaluation of the graft.

- In 4 of 12 cases the monkeys disturbed their grafts leading to death in three of those cases. For this model to be successful, modifications need to be implemented that can reduce the occurrence of graft disturbance.
- The mismatch in graft diameter compared with the vessels of the monkey may increase the probability of thrombosis in this model.
- The significant length of graft positioned subcutaneously in the groin area may be prone to bending, kinking and external pressures during the course of daily activity, particularly as the monkeys tend to spend a lot of time in a crouched position.
- The 6-8cm length of graft placed in such a small space necessitates a graft loop with a very small diameter (~1cm) that may create hemodynamics that initiate thrombosis.
- Since the graft segments were relatively long, it was necessary to place the grafts perpendicular to the anastomoses while a 30 degree angle of anastomosis would have been more optimal for smooth blood flow to avoid turbulence that can activate platelets and thrombotic events.

Possible improvements for the model include:

- Implementation of safeguards to prevent animals from disturbing the grafts. The most significant change to incorporate is modification to the method of closure. Closure of the surgical site should be completed in layers including subcuticular and intradermal sutures and only interrupted stitches should be used.
- A less aggressive model might be possible using a much shorter segment of the graft in a straight (not looped) configuration with angled anastomoses. The utility of the model for assessing kinking, cannulation, and repopulation and endothelialization would be limited. With the limited size of the monkey thigh, it is not clear that this model is feasible.

Conclusions

This study was designed to use the African green monkey as a model to study the human umbilical vein graft. This study demonstrates that the umbilical vein graft (UVG) can be implanted as an arteriovenous shunt from the iliac artery to the femoral vein in this primate and remain patent for as long as six weeks. The data show that there is a humoral immune response to the graft in this model. However, despite this response, no mural degeneration or aneurysms in the graft were noted within the first six weeks. An initial inflammatory reaction to the graft was shown to resolve over time giving way to a healing response and the initiation of new tissue growth. Taken together these results indicate that although this is a xenogeneic model (Human to Monkey), it may provide a means of obtaining information about the expected function, hemocompatability and healing response of the UVG for as long as six weeks *in vivo*. This study also demonstrated that the graft can be cannulated in the chosen position and that hemostasis after cannulation of the UVG compares favorably to ePTFE.